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# Modification of a Collagen Scaffold to Enhance the Performance of Tissue Engineered Skin

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# Modification of a Collagen Scaffold to Enhance the Performance of Tissue Engineered Skin

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A Major Qualifying Project Report  
submitted to the Faculty of  
Worcester Polytechnic Institute  
in partial fulfillment of the requirements for the  
Degree of Bachelor of Science  
By

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Keywords:  $\mu$ DERM, FGF-2, heparin, angiogenesis

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## Abstract

To address the limitations of current commercially available tissue engineered skin substitutes, Clement et al has created a novel micro-patterned dermal-epidermal regeneration matrix ( $\mu$ DERM). This scaffold consists of a micro-patterned collagen gel coupled to a collagen sponge to replicate the dermal-epidermal junction of the skin. However, the ability of the scaffold to promote vascularization has not been assessed. In order to promote  $\mu$ DERM vascularization, the team added Fibroblast Growth Factor – 2 (FGF-2) and heparin to the collagen sponge. Additionally, the team produced an *in vitro* model to assess the efficacy of the enhanced system. To evaluate the effects of these factors on cellular outgrowth, the team designed a system that allowed for efficient analysis of the outgrowth of NIH/3T3 Fibroblast cells towards the collagen-GAG sponge. Six different conditions were tested: 1) Non-heparinized with no FGF-2, 2) Heparinized with 100 ng/mL FGF-2, 3) Heparinized with 200 ng/mL FGF-2, 4) Heparinized with 300 ng/mL FGF-2, 5) Non-heparinized with 300 ng/mL FGF-2, and 6) Heparinized with no FGF-2. PDMS molds that allowed for uni-directional movement of the cells along rectangular channels were fabricated for each individual well of a 6-well plate. Image analysis was done over a period of twenty-four hours to obtain quantitative results. The fibroblast outgrowth rates were highest when exposed to 300 ng/mL of FGF-2 compared to a non-heparinized, non-growth factor stimulated scaffold. These results can most likely be attributed to the initial burst of growth factor. Overall, the addition of heparin alone did not show significant effect on fibroblast outgrowth rates, and thus it is suggested that a future study collects data over a longer period of time to understand the potential long-term angiogenic effect that heparin could have on the scaffold.

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## 1.0 Introduction

Modern day tissue engineering is driven by the crucial need for effective, long lasting chronic wound treatments. In the United States, over 6.5 million patients each year suffer from chronic wounds and need immediate medical attention (Sen et al., 2009). Additionally, over 450,000 individuals are hospitalized each year for life-threatening burns (*Burn incidence and treatment in the United States*, 2012). Chronic wounds and chronic burns do not heal in the orderly way that most wounds do (Sen et al., 2009). Current treatment methods for chronic burns and wounds can be costly and ineffective. Wound inflammation and infection, patient discomfort, and repeat procedures are all adverse effects of clinical treatment methods (Sen et al., 2009). Thus, the crucial need for reconstructed tissue engineered skin substitutes with reduced side effects and improved efficacy is shown through these adverse issues.

The current standards of care for large area full-thickness skin wounds include autografts, allografts and tissue engineered skin substitutes. The preferred treatment method for large area skin wounds is autografting. This process consists of transplanting large portions of a patient's skin from one area of the body and relocating it to the wound site. However, autografts are limited in cases where patients possess large burns or skin traumas because there is a lack of healthy skin to be used as a donor site for transplantation. Allografting is the transplantation of same-species donor skin. Allografts are used for temporary wound coverage in patients with large wounds, but do not suffice for long term treatment because of problems with rejection and dermal growth (Kamel et al., 2013). Similarly to autografting, autologous cell culture involves taking a small biopsy of healthy tissue from the patient and allowing it to culture into transplantable sheets. However, autologous grafting requires long periods of time to produce sufficient amounts of skin (Kamel et al., 2013). Additionally, autologous grafting increases

susceptibility to infection with decreased efficacy through the presence of collagenase enzymes located within the wound (Kamel et al., 2013). Now, synthetic skin substitutes are being explored as cellular, acellular, or composite products. Synthetic substitutes are stable, biodegradable three dimensional structures composed of immunocompatible materials (Halim et al., 2010). Examples of commonly used engineered skin applications are Apligraf, Integra, and Dermagraft. No single product, however, has been proven fully effective for all cases due to high costs and the need for additional surgeries (Kamel et al., 2013). Also, synthetic structures lack sufficient basement membranes and are not as architecturally stable (Halim et al., 2010). These skin grafting limitations prompt the need for improved, long-lasting, tissue engineered skin substitutes and corresponding application methods.

The goal of this project is to enhance angiogenesis of a tissue engineered skin substitute through modification of a pre-existing scaffold called micro-patterned dermal-epidermal regeneration matrix ( $\mu$ DERM). Currently  $\mu$ DERM consists of two main layers, the top a micro-patterned collagen gel and the bottom consisting of collagen sponge. The collagen gel is cast onto micro-patterned molds to create micro-niches onto the gel surface (Clement et al., 2013). However, the ability of the  $\mu$ DERM to promote vascularization has not yet been assessed. Thus, the team modified the  $\mu$ DERM to enhance angiogenesis and tissue ingrowth. Additionally, the team produced an *in vitro* model to assess the efficacy of the enhanced system.

## 2.0 Literature Review

### 2.1 Clinical Significance

The ample prevalence of chronic wounds, severe burns, and additional preceding diseases creates a need for tissue engineered skin substitutes (Sen et al., 2009). In the United States alone,

over 6.5 million patients annually experience chronic wounds that need immediate medical treatment (Sen et al., 2009). These chronic wounds can stem from pre-existing diseases like ulcers, obesity, and diabetes (Sen et al., 2009). In addition, roughly 450,000 individuals in the United States are hospitalized as victims of severe burning (*Burn incidence and treatment in the United States*, 2012). Individuals suffering from severe burns need immediate medical treatment to assist in the skin regeneration process. Current treatment methods for severe burns and chronic wounds are ineffective due to high costs, time constraints, and the need for multiple surgeries (Atiyeh et al., 2005). Ineffective treatment methods can result in tissue infection, inflammation, or patient morbidity (Atiyeh et al., 2005). Tissue engineered skin substitutes and application methods with improved efficacy and longevity are necessary to efficaciously treat affected individuals.

### 2.1.1 The Importance of Skin

To understand the mechanisms of wound healing, the structure of healthy skin must first be examined. Skin is the largest organ in the body and acts as a crucial physiological defense mechanism by protecting the body from foreign microorganisms, environmental toxins, and ultraviolet radiation exposure (Venus et al., 2011). The three primary constructs of skin- the epidermal layer, dermal layer, and subcutaneous tissue- provide structural support through different components and functions (Mikesh et al., 2013).

The epidermis is the outermost layer of skin and contains stratified cellular layers of keratinocytes, Langerhans cells, and melanocytes (Kanitakis, 2002). The epidermis consists of four major regions: basal cell layer, spinous cell layer, granular cell layer, and horny layer (Venus et al., 2011). Each layer aids in the differentiation and repair processes of epidermal cells (Kanitakis, 2002). In the basal layer, approximately 30% of epidermal cells will divide to

perform two specific functions (Powell, 2006). Epidermal cells will continue to divide in the basal layer while the remaining travel through the spinous, granular, and horny layers to differentiate into keratinocytes (Powell, 2006). Differentiated keratinocyte cells will release at the horny layer, beginning the skin shedding cycle. This cycle occurs every 28 days in healthy patients (Powell, 2006).

The dermal layer of skin is comprised of elastic connective tissues (i.e. collagen and elastin fibers) and provides protection against physical injury or trauma to the epithelial layer (Kanitakis, 2002). Additionally, the dermal layer consists of collagen and elastin fibers which provide structural support, integrity, resilience, and elasticity to the skin (Venus et al., 2011). The three main cell types found in the dermal layer- fibroblasts, macrophages, and mast cells- play an important role in the wound healing cascade and tissue regeneration process (Kanitakis, 2002).

The innermost and deepest layer of skin is subcutaneous tissue, or fatty tissue. Adipocytes are the fundamental cell type found in subcutaneous tissue. These cells are responsible for ensuring insulation, temperature regulation, protection from injury or trauma, and energy storage (Kanitakis, 2002).

### 2.1.2 The Importance of Proper Wound Healing

Cutaneous wound healing is comprised of three overlapping stages: inflammation, tissue-formation, and tissue-remodeling. Factors affecting the rate of wound healing include location and depth of the wound and overall health of the patient. It is important for all stages of wound healing to be complete for maximum recovery.

Inflammation, the first step in the wound healing process, begins when platelets form a blood clot and release a specific factor that attracts macrophages and fibroblasts. While neutrophils aid in the disposal of debris, macrophages help transition the phase of inflammation

into tissue formation with expression of colony-stimulating factor 1, tumor necrosis factor, platelet-derived growth factor (PDGF), transforming growth factor alpha and beta (TGF-alpha and TGF-beta), interleukin-1, and insulin like growth factor I. (Clark et al., 1999)

Tissue formation begins one to two hours after inflammation. This allows lateral movement of epidermal cells from the wound margins, and later reattachment once the basement membrane is reestablished (Clark et al., 1999). Lateral movement of the epidermal cells creates a barrier between live tissue and the fibrin clot now present in the wound. Beneath the moving epithelial cells, new epidermal cells proliferate and basement membranes form from the deep tissue from the outer edge of the wound to the inner. Kucharska et al. have identified EGF, TGF-alpha, and keratinocyte growth factor as potential growth factors that may cause this transition. (Kucharska et al., 2011)

Neovascularization and the formation of granulation tissue are the next two steps in the tissue remodeling process. Granulation is the transitional process of the provisional extracellular matrix turning into the collagenous matrix, which occurs about four days after inflammation. Macrophages, blood vessels, and fibroblasts contribute significantly to this transition. Macrophages promote angiogenesis via growth factors, and blood vessels form the capillaries in the stroma that carry the nutrients needed for continued cell growth. Fibroblasts produce collagen, which forms the new extracellular matrix which allows cell ingrowth into the wound (Guo et al., 2010). The migration of fibroblast cells into the wound is rate limited. The tissue ingrowth and repair process can also be prohibited by integrin receptor binding. Integrin receptors bind fibronectin and fibrin to fibroblasts. If the necessary integrin receptors are not present, or fibronectin is slow to bind to fibroblasts, the fibroblasts will not produce collagen. Some growth factors that aid in fibroblast proliferation and expression of integrin receptors



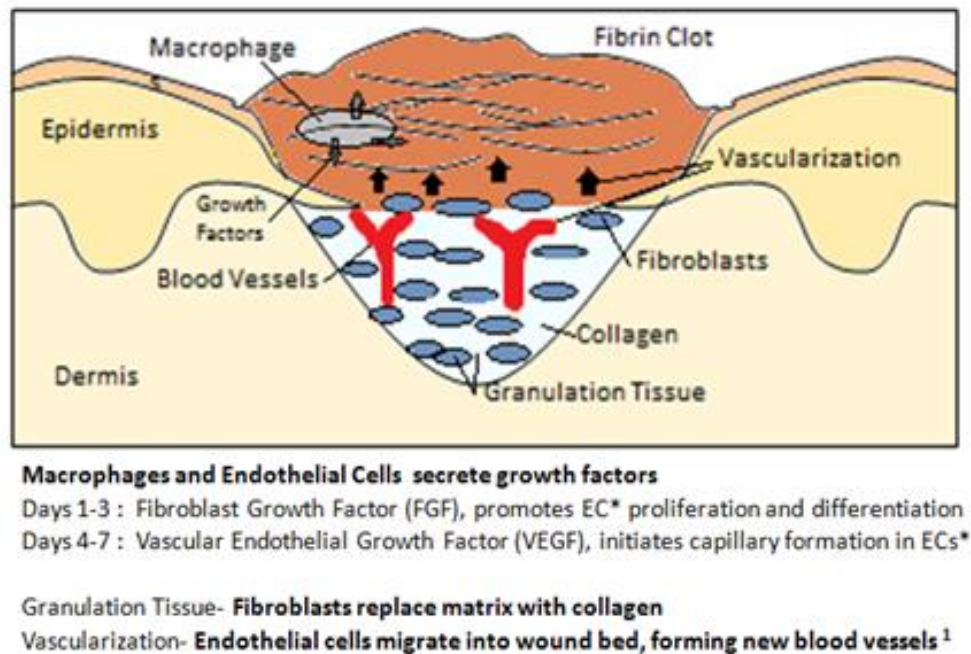
include TGF- beta and PDGF. To summarize, factors that slow the formation of granulation tissue are integrin receptors and the level of cross-linking in the extracellular matrix.

Plasminogen activator, collagenases, gelatinase A, and stromelysin are used to cleave a path for cell migration. (Clark et al., 1999)

Neovascularization supports tissue granulation by facilitating angiogenesis. Angiogenesis is when endothelial cells are stimulated by growth factors and form new blood vessels at the site of injury. Endothelial cells release plasminogen activator and procollagenase. This release is stimulated by FGF-2. The plasminogen activator causes plasminogen to convert to plasmin and activates collagenase. The plasmin and collagenase digest the basement membrane (Clark et al., 1999). This allows for the activated endothelial cells to move into the wound site and create new blood vessels. Once this process is complete, many cells and blood vessels are dissipated via apoptosis. Factors promoting blood vessel formation include fibroblast growth factor (FGF-2), TGF-beta, angiogenin, angiotropin, angiopoietin 1, thrombospondin, low oxygen tension, and elevated lactic acid. Macrophages and endothelial cells produce FGF-2 and vascular endothelial growth factor (VEGF); FGF-2 is used in the first three days of neovascularization and VEGF is needed in days four through seven. VEGF production can also be stimulated by hypoxia of the wound. Proteolytic enzymes can increase macrophages at the site of injury, also enhancing natural growth factors needed for angiogenesis. (Clark et al., 1999)

The last step of tissue-remodeling occurs during the second week of healing with wound contraction and extracellular matrix reorganization. Wound contraction occurs when myofibroblasts, stimulated by TGF-beta one or TGF-beta two and PDGF, attach to the collagen matrix. The remodeling of collagen during this time is dependent on the rate of synthesis of fibrillar collagen, contraction of the wound, the rate of formation of larger collagen bundles, and

an increase in cross-links. After approximately three weeks the wound will have 20% tensile strength and after several months to a year the wound will gain 70% tensile strength (Clark et al., 1999). Figure 1, adopted from Clark et al. demonstrates the components of vascularization as discussed above.



*Figure 1: Summarizes the discussed wound-healing response in terms of the granulation and vascularization stages of wound healing. (Clark et al., 1999)*

## 2.2 The Need for Skin Substitutes

There is a large clinical need for skin substitutes which provide healing for large, full-thickness wounds. Wounds that are extremely deep and damage the dermal layer of the skin do not allow proper regeneration, as extensive damage has been done to essential regions of the dermis. Substitutes allow for immediate wound coverage and can stimulate the release of growth factors and cytokines to promote keratinocyte proliferation, fibroblast proliferation, and endothelial cell migration. The anatomical purpose of receiving tissue engineered skin is to

stimulate natural responses in the body while closely mimicking the anatomy of physiological skin with native mechanical and biological properties. Successful transplants of skin substitutes will result in no host toxicity or immune rejection (Kamel et al., 2013). For instance, chronic foot ulcers elicit multiple pathological factors like vascular insufficiency, altered cellular activity, and a dysfunctional extracellular matrix. These pathological factors impair proper healing and lead to an altered wound bed and failed response to conventional therapy alone. The goal of tissue engineered substitutes is to augment this healing process and trigger biological effects that allow for proper wound healing. Currently, many techniques and products have been developed in attempt to find a solution for successful treatment of full-thickness wounds.

## 2.3 Current Skin Grafts

Skin grafts for treatment of wounds can be compiled into two prominent groups: biological and tissue engineered. Biologicals involve taking natural skin tissue and transplanting it onto the patient's wound area. Tissue engineered skin grafts have a plethora of varieties and provide an alternative to the standard split-thickness grafts obtained in biological grafts. Tissue engineered grafts come in many forms, some solely cover the epidermal layer, some solely attempt to replace the dermal layer, and some are composites that attempt to regenerate the entire infrastructure of the dermal and epidermal layers (Kamel et al., 2013). It is important to examine multiple facets of biological and tissue engineered grafts so that their advantages and limitations can be fully understood.

### 2.3.1 Biological Tissues

Autografts are one of the current standards of care for wound healing of patients with serious tissue damage related to the skin. Autografting is the process in which a section of a patient's healthy skin is surgically removed and transplanted onto the wound area. Since these

grafts are taken from the patient's own healthy tissue, they provide the natural cells and mechanical structure to help stimulate tissue growth. These grafts integrate with the damaged tissue without causing an immune response in the patient (rejection) (Balasubramani et al., 2001). Autografts also have been proven to provide reasonable cosmetic results due to the lack of scarring in the wound bed (Singer et al., 1999).

Allografts, or "donor skin" from healthy patients or cadavers, were developed in the early 1980s to help avoid the problem of treating large wound sites and creating second wound sites when performing autografts. Allografting technique includes seeding epidermal cells on a culture plate and allowing them to grow, which is beneficial because it makes preparation faster and easier. These skin substitutes are used to stimulate the host keratinocytes to proliferate through the release of cytokines to heal the wound. Due to this phenomenon, allografts are not appropriate for full-thickness wounds like autografts, but can be used for temporary wound coverage (Balasubramani et al., 2001).

Cultured composite autografts (CCA) are another form of natural grafting that can be implemented by surgeons. These grafts are composed of cultured keratinocytes and fibroblasts that are obtained through biopsies of the patient at multiple sites. Both cell types are cultured separately during the initial phases of growth. After nine days, the keratinocytes are cultured over the fibroblasts, causing the formation of a multilayered epidermal component and an extracellular dermal component through the secretion of proteins and glycoproteins. After the CCA cultures for 16 days, a histological examination will show stratified squamous epithelium overlying a dermal bed with a type IV collagen basement membrane. This provides the surgeon with a substantial neo-dermis to be grafted onto the patient (Caruso et al., 1999). Although this system mimics the infrastructure of the epidermal and dermal layers, it does not promote

vascularization (Caruso et al., 1999). Composite grafts can be considered a model system to engineered skin grafts.

### 2.3.2 Engineered Tissues

Although there is a moderate array of tissue engineered skin products on the market, each has particular advantages and disadvantages to its application. Products can be either cellular, acellular, or a composite of both, and can contain a dermal, epidermal, or combination of components. Cellular tissue engineered products use living cells such as fibroblasts or keratinocytes as the main component of a matrix or scaffold, while acellular products use a matrix or scaffold composed largely of materials like collagen or fibronectin. Dermal components in tissue engineered skin products are intended to prevent wound contraction and provide greater mechanical stability. Epidermal components could be responsible for wound coverage and closure, and may be responsible for cosmetic outcome. It is useful to focus on a select few tissue engineered skin products and compare their characteristics. There are currently three that appear to be most common for clinical use: Apligraf, Dermagraft, and Integra (Kamel et al., 2013).

Engineered grafts can also be fabricated biologically by culturing cells from the patient or a donor site. Similar to autografts, autologous tissue engineered grafts utilize a patient's own cells that can be grown into skin sheets to provide extensive wound coverage on victims with a large burn percentage on their body. For autologous tissue engineered grafts to be fabricated, a stamp-sized biopsy is taken from a patient's healthy skin and is cultured to produce healthy epidermal sheets. Although effective, the limitations of engineered tissues will be discussed in section 2.4.3.

#### 2.3.2.1 $\mu$ DERM Description

Clement et al have developed a composite skin substitute called the Micro-patterned Dermal-Epidermal Regeneration Matrix ( $\mu$ DERM). This tissue engineered product utilizes type I collagen, neonatal primary human foreskin fibroblasts (NHFs) and primary human foreskin keratinocytes (NHKs) to imitate the complex interface of the dermis and epidermis and serve as a full-thickness wound regeneration platform. It employs micro-niche topography to influence cellular behavior and subsequently enhance epidermal morphology and thickness. The following process is used to fabricate this matrix: 1) a silicon wafer is created with micro-niches resembling the dermal-epidermal junction (DEJ) and serves as a master pattern, 2) PDMS is cast on this pattern to create a negative mold onto which type I collagen is assembled, forming a collagen gel 3) a collagen-GAG sponge is laminated to the collagen gel, 4) the complete matrix is cross-linked for enhanced mechanical stability. The collagen-GAG sponge represents the dermal side while the micro-patterned gel represents the epidermal surface. Following the fabrication of  $\mu$ DERM, the dermal side is seeded with fibroblasts, and the epidermal side with keratinocytes. Clement et al notes that the micro-patterned tissue analog is implantable, and thus it has potential for significant *in vivo* success. (Clement et al., 2012)

#### 2.3.2.2 Apligraf®

Apligraf® is a tissue engineered skin substitute currently on the market to treat diabetic foot ulcers and venous leg ulcers. Similarly to human skin, Apligraf® consists of living cells and structural proteins. The lower dermal layer combines bovine type 1 collagen and allogeneic human fibroblasts (dermal cells), which produce additional matrix proteins. The upper epidermal layer is formed by promoting allogeneic human keratinocytes (epidermal cells) first to multiply and then to differentiate to replicate the architecture of the human epidermis. Apligraf® is also

the only living, bi-layered cell based product FDA approved for use in these clinical settings. (Streit et al., 2000)

### *2.3.2.3 Dermagraft®*

Dermagraft® can be classified as a cellular, dermal tissue engineered skin product used to treat diabetic skin ulcers (Kamet et al., 2013). Dermagraft® is cryopreserved, three dimensional, and acts as a dermal substitute (Kamel et al., 2013). It is composed of human fibroblasts, an extracellular matrix, and a bioabsorbable polyglactin mesh scaffold. Dermagraft® helps to restore the compromised dermal bed to facilitate healing by providing a substrate over which the patient's own epithelial cells can migrate to close the wound. (Halim et al., 2010)

### *2.3.2.4 Integra®*

Integra® is an acellular, semi-permanent collagen-silicone matrix used in over 10,000 patients for treatment of chronic burns, diabetic ulcers, and auricular reconstruction (Hart et al., 2012). It contains a dermal component composed of bovine collagen and shark cartilage-GAG that promotes tissue ingrowth of both fibroblasts and keratinocytes, while an epidermal component made of silicone is responsible for wound closure and prevention of fluid loss. The dermal component enhances ingrowth of fibroblasts. (Hart et al., 2012)

## *2.4 Limitations of Current Skin Grafts*

### *2.4.1 Limitations of Biological Tissues*

As mentioned before, autografts and autologous cultured grafts provide coverage and immediate treatment of wounds to stimulate keratinocyte proliferation for wound healing. Although they provide an outer layer of skin that protects the body from infection, there are two major limitations to this standard of care (Balasubramani et al., 2001). The first, lesser important, major limitation to this type of procedure is the time it takes to culture a significant amount of

skin for coverage of large wounds. The second foremost limitation to this standard is the inability of these grafts to integrate with the patient's wound to promote tissue in growth and vascularization. Similarly, allografts, which can be stored and produced in large quantities to avoid problems with autografts, have disadvantages as well. One limitation to allografts is that they only provide temporary wound coverage by merely stimulating host keratinocytes to proliferate (Kamel et al., 2013). This makes them unsuitable for treatment of full-thickness wounds because of their lack of ability to integrate with the dermal layer of skin. These skin grafts can also cause an immune response if the body rejects them as foreign (Kamel et al., 2013). Neither grafting technique provides adequate angiogenesis as they do not sufficiently stimulate migration and differentiation of endothelial cells to trigger blood vessel growth and wound bed integration (Kamel et al., 2013). These major problems have led to research of other potential techniques to provide angiogenesis and new tissue ingrowth for full-thickness wound injuries.

#### 2.4.2 Limitations of Engineered Tissues

Currently, engineered skin substitutes come in a variety of forms. They are primarily categorized into three classes: (I) temporary impervious dressing materials, (II) single layer durable skin substitutes, and (III) composite skin substitutes (Halim et al., 2010). Dressing materials are single layer materials with the purpose of providing temporary wound closure, a barrier to bacteria, and a moist environment. Single layer substitutes serve as substitutes for either the dermal or epidermal layer alone, while composite substitutes aim to mimic the joint relationship of the dermis and epidermis (Halim et al., 2010).

Like biological skin substitutes, tissue engineered skin products also have limitations. Kamel et al has recently published a review to comment on the current state of tissue engineered



products, including advantages and disadvantages for the majority – if not all – of the tissue engineered products commercially available; Apligraf®, Dermagraft®, and Integra® are included in this review (Kamel et al., 2013). Some limitations of Apligraf® include: required multiple applications, cells in the construct do not survive after 1-2 months *in vivo*, it poses a short shelf life, is difficult to handle, poses risk of disease transfer, and is manufactured at high costs. Limitations of Dermagraft® include: multiple applications are required, there is a chance of disease transfer or rejection, and it is costly. Integra®’s limitations primarily stem from its significant preparation time; three weeks are required for preparation prior to application, and thorough surgical preparation of the wound bed is necessary to guarantee successful graft take (Kamel et al., 2013). Most significantly, commercially available tissue engineered products do not yield adequate angiogenesis after implantation. Limitations to these products provide insight as to what must be considered when constructing an improved skin substitute.

## 2.5 Innovations to Current Technology

A major factor in skin engineering is integration of engineered skin with native surroundings. “Currently, the success of tissue engineering skin is very dependent on the skillful use of surgical techniques and preparation of the wound bed” (Mohamed et al., 2012). Increasing the success of graft take in synthetic tissues is complex. Current engineered skin does not sufficiently promote tissue ingrowth and vascularization. Inadequate tissue ingrowth causes graft failure because native tissue migration into the engineered skin scaffold is insufficient. Also, a lack of tissue vascularization causes graft failure because oxygen and nutrients will not properly transfer to the bottommost layer of the skin graft. It is logical to question the functions of increased tissue ingrowth and vascularization in graft take of engineered skin.

### 2.5.1 Endothelial Cells in Tissue Ingrowth and Vascularization

As discussed in previous sections, endothelial cells migrate into the wound site after fibroblasts form a collagenous matrix in which endothelial cells may be guided in their migration. Once in the wound bed, endothelial cells form new blood vessels in the process of angiogenesis. Endothelial cell migration can be described in six stages: sensing, extension, attachment, contraction, release rate, and recycling (BurrIDGE et al., 1996). Sensing occurs when filopodia (long tapering pseudopodium) extend in search of the VEGF gradient. Extension follows as protrusion of the leading edge of the endothelial cell ensues. In the attachment phase, protrusions attach to the focal adhesions of the extracellular matrix (ECM). A focal adhesion is a form of tight adhesion under the ECM that provides structure, which links the ECM to the actin cytoskeleton. Focal adhesions are also areas that allow for signal transduction, which controls growth (BurrIDGE et al., 1996). Next, contraction occurs with stress fiber formation and induced cell contraction allowing for forward movement of the cell. This is followed by release of the endothelial cell via focal adhesion disassembly. Finally, adhesive and signaling components are recycled (Lamalice et al., 2007).

Three mechanisms affect endothelial cell migration: chemotactic, haptotactic, and mechanotactic stimuli. Chemotaxis is endothelial cell migration toward chemoattractants like growth factors. Haptotaxis is endothelial cell migration toward immobilized ligands, which increases integrin binding to the ECM. Mechanotaxis is endothelial cell migration from mechanical forces, mainly shear stresses (Lamalice et al., 2007). Chemotactic stimuli, VEGF and FGF-beta, enhance and activate haptotactic stimuli by affecting integrins  $\alpha v\beta 3$ ,  $\alpha v\beta 5$ , and  $\alpha 5\beta 1$  (Byzova et. al, 2000).

Endothelial cell migration is vital to angiogenesis (Lamallice et al., 2007). Once endothelial cells migrate into the wound bed, they are stimulated by growth factors, reactive oxygen species (ROS), and other stimuli to form blood vessels at the site of injury.

### 2.5.2 Current Factors for *In Vitro* Angiogenesis in Skin

Growth factors which are crucial to angiogenesis in cutaneous wound healing include epidermal growth factor (EGF), FGF-2, TGF-beta, and PDGF. EGF is widely used as a mitogen for epithelial cells and accelerates wound repair via enhanced formation of granulation tissue and increased collagen content. Collagen can be coupled with FGF-2 and TGF-beta for promotion of full-thickness wound healing by increasing the rate of epithelialization, contraction, and blood vessel formation (Amankwah et al., 2007). Additional growth factors include VEGF and platelet-derived growth factor-BB (PDGF-BB). When these factors are combined into a scaffold, VEGF is released faster and PDGF-BB is released slower. Together, VEGF and PDGF-BB support fibroblast growth, promote angiogenesis, increase re-epithelialization, and control granulation tissue formation. In the later stages of wound healing, the factors promote quicker collagen deposition and earlier remodeling of the injured site for faster healing (Xie et al., 2013). Anti-inflammatory macrophages (M2) promote angiogenesis. Studies have shown increased numbers of endothelial cells and tubular structures in the presence of M2 macrophages, which induce expression of FGF-2, insulin-like growth factor-1 (IGF1), chemokine (C–C motif) ligand-2 (CCL2) and placental growth factor (PGF) (Jetten et al., 2014).

### 2.5.3 Novel Factors for *In Vitro* Angiogenesis in Skin

High levels of ROS are found in tumors and can have an effect on angiogenesis. ROS are regulated by endogenous antioxidant enzymes like superoxide dismutase (SOD) and thioredoxin. In endothelial cells, a major source of ROS is nincotinamide adenine dinucleotide phosphate

(NADPH) oxidase which is activated by VEGF, angiopoietin-1, hypoxia, and ischemia. ROS are involved in VEGF receptor-2 (VEGFR2), autophosphorylation, and redox signaling pathways that stimulate transcription/genes that promote angiogenesis. There is still more work being done on ROS in relation to angiogenesis (Ushio et al., 2008).

In addition, research is being done on new complementary cell co-cultures to explore the effects of multi-cell stimulation of growth factors. Two cells specifically being examined for co-culture are mesenchymal stem cells (MSCs) and Muller cells. MSCs have been reviewed in cardiac tissue treatment because of their ability to promote angiogenesis and neurogenesis. In specific relation to skin, MSCs may be co-cultured with endothelial cells to increase wound healing properties for skin; specifically, MSCs from Wharton's jelly promote microvasculature formation and cell migration on co-cultured endothelial cells (Hsieh et al., 2013). Muller Cells in retinal tissue when under conditions of hypoxia stabilize hypoxia-inducible factor-1 alpha (HIF-1 alpha) and secrete angiogenic cytokines, promoting vascular permeability. Muller cells require HIF-1 alpha, but do not require VEGF. When applied to endothelial cells, the blocking of HIF-1 alpha inhibited vascular permeability (Xin et al., 2013).

Differing vascularization rates and efficiency can also be seen in factors that are chemically manipulated. An example of this is heparin, an anti-coagulant found in the liver. Heparin is chemically bound to polymer scaffolds and interacts with heparin-binding angiogenic growth factors including VEGF and FGF-2. Heparin is not directly a wound healing factor, but it is a chemical that attaches to the scaffold and directly interacts with the factors to promote factor regulation and release. By monitoring the rate at which these factors are distributed, the effects of the factors are enhanced over a longer period of time and this increases angiogenic potential. (Amankwah et al., 2007)

An additional novel option for up regulation of vascularization is microRNA, or noncoding RNA. Endothelial cell-specific microRNA-126 promotes angiogenesis when growth factors VEGF and FGF-2 are delivered to the wound bed. By increasing the microRNA-126 presence, the effects of a given amount of growth factor may increase without increasing the quantitative amount of the factor. (Fish et al., 2009)

#### 2.5.4 *In Vitro* Controlled Release Mechanisms for Growth Factors

Growth factors can significantly enhance angiogenesis of tissue engineered skin into the wound bed. However, in order to apply a growth factor or combination of growth factors, a controlled release mechanism is needed. Controlled release mechanisms allow for the growth factor to be released at a sustained rate that best mimics the stages of wound healing and facilitates the tissue regeneration process. Growth factors can be applied through bulk encapsulation, specific or non-specific surface adsorption, and biodegradable microsphere encapsulation. Studies have shown that the release of paracrine factors in a local and sustained manner may greatly enhance tissue remodeling and organogenesis. (Chung et al., 2007)

##### 2.5.4.1 *Embedding Growth Factors within a Porous Scaffold*

One approach to sustained delivery of growth factors is to simply embed the factor during the fabrication process of a porous scaffold. The incorporation of angiogenic growth factors has been done by incorporating the factor into the polymeric solution prior to fabrication. This can be achieved by mixing the protein powder with polymer particles followed by cross-linking. This process has been seen to exhibit sustained release of the growth factor VEGF, to promote cellular proliferation of endothelial cells *in vitro* and angiogenesis *in vivo* (Chung et al., 2007). In order for this method to be effective, the porous scaffold must undergo some degradation to allow for release of the embedded factor (Tabata et al., 1998). Modifying porous collagen-GAG

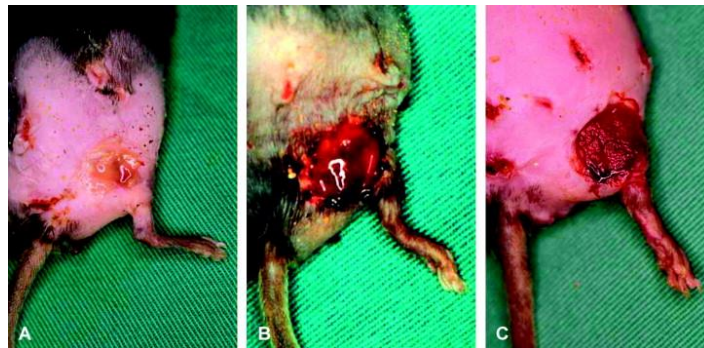
scaffolds cross-linking levels could potentially allow for an angiogenic factor to be directly incorporated into the collagen slurry and released at a controlled rate.

#### *2.5.4.2 Using Biodegradable Microspheres for Delivery*

Similar to embedding growth factors into the scaffold, biodegradable microspheres can be used to deliver growth factors at a controlled rate by combining them within a scaffold. One of the most common techniques used to achieve this is the use of poly(lactic-co-glycolic acid) (PLGA) microspheres to encapsulate the growth factor of choice. PLGA microspheres have good biodegradable properties as varying the ratio of PLA to PGA can result in a wide range of degradation rates. Similarly to embedding growth factors, these microspheres can be embedded within the scaffold during its fabrication process. These microspheres have been used in previous applications including entrapment within an alginate scaffold to deliver FGF-2 to induce proliferation of cardiofibroblasts *in vitro* and angiogenesis *in vivo*. However, some of the common issues with this method are the maintenance of physical integrity of the scaffold and bioactivity of the proteins (Chung et al., 2007).

Another commonly used method for delivery of growth factors are gelatin microspheres. Gelatin microspheres are biodegradable and can be impregnated with growth factors such as FGF-2 for controlled release. These microspheres are fabricated through a process of glutaraldehyde cross-linking of gelatin aqueous solution. They can then be loaded by dropping a volume of aqueous solution of FGF-2 onto dried microspheres and allowing them to sit at room temperature and impregnate themselves. In a previous study, gelatin microspheres containing FGF-2 were implanted into the subcutaneous region of a mouse model that was induced with a diabetic ulcer (Kawai et al., 2005). *In vivo*, angiogenesis in the mouse was much more

prominent in FGF-2 loaded microspheres as compared to FGF-2 applied at a single dose and a control containing no FGF-2 as seen in Figure 2 (Kawai et al., 2005).



*Figure 2: Shows the in vivo angiogenesis in a nude mouse model after 5 days of implantation. Image A shows the control of only gelatin microspheres alone, image B shows capillary formation in the presence of only FGF-2, and image C shows capillary formation in the presence of FGF-2 loaded gelatin microspheres. (Kawai et al., 2013)*

Angiogenesis was quantified by both fibroblast proliferation and capillary growth. At day 5 of implantation, it was seen that the control group and single dosed FGF-2 group became less effective than in the first stages of healing. However, FGF-2 released at a controlled rate using gelatin microspheres continued to show fibroblast proliferation and capillary growth into day 10 of healing. This study also proved that gelatin microspheres can be injected into the inner portions of porous collagen sponges for possible use in tissue engineered skin scaffolding (Kawai et al., 2005). One of the first steps of capillary and blood vessel formation *in vivo* is the migration of endothelial cells (Lamalice et.al, 2007). Although this study did not quantify endothelial cell migration, it can be hypothesized due to the extensive formation of capillaries *in vivo* that FGF-2 did have a large effect on the migration of endothelial cells.

#### *2.5.4.3 Immobilization of Scaffolds Using Heparin Engraftment*

FGF-2 has been seen to have a large impact on fibroblast proliferation, neovascularization, osteogenesis, and nerve regeneration. Heparin is a highly sulfated

glycosaminoglycan that has the largest negatively charged density of any known biological molecule allowing it to bond to a variety of positively charged molecules. Use of heparin on tissue regenerative scaffolds has been seen to be extremely effective as growth factors, including FGF-2, have a very high bonding affinity to it. This is very important as it protects the growth factor from protein denaturation and enzymatic degradation. It has been seen to be effective within PLGA microspheres as well collagen matrices (Ho et al., 2009). Heparin also has been seen to significantly enhance the controlled release of growth factors due to the presence of a reversible thermodynamic equilibrium between the immobilized heparin and the incorporated growth factor. Studies have combined immobilized heparin with collagen through a conjugation reaction between carboxyl groups on the heparin and amino groups on the collagen. Studies have shown that combining heparin with both VEGF and FGF-2 had a large increase in protein bioactivity to promote angiogenic activity (Chung et al., 2007). Heparin could be a very important factor in the advancement of scaffolds to enhance skin regeneration.

## 2.6 Proposed Contribution to the Field

The team hypothesized that mimicking parts of granulation and neovascularization in natural skin will allow for improved graft take of engineered skin. Improved graft take will include the ingrowth of endothelial cells into the scaffold. The increase in tissue ingrowth will create a stronger bond between the basement membrane and bottom of the dermal scaffold through increased endothelial cell migration and increased vascularization. An increase in blood vessel growth between the native tissue and scaffold will allow for increased circulation of nutrients and oxygen, which will make the tissue and cells connecting the scaffold to its native surroundings stronger. Additionally, a system that provides tunable regulation of factors affecting tissue ingrowth and vascularization will allow quantification of the relationship



between graft take and native tissue response. These improvements to the field of skin grafts will aid patients by producing engineered skin with enhanced graft take components similar to that of native tissues.

## 3.0 Project Strategy

### 3.1 Design Process

Before objectives, functions, and constraints can be established, it was important to understand whom is fulfilling each role of the project. The three stakeholders include clients, users, and designers. The client played one of the major roles in the design process as they established the initial idea and statement defining the ultimate goals for the design. In this case, Professor George Pins was the client, as he was the driving force behind the eventual direction the project progressed. The next important stakeholder of the project was the user. The user helped drive a different section of the project which was described as design usability and efficacy. If the design was not efficient or easy to use, the user would not want to invest in the design. It was important to establish Amanda Clement as the ultimate user of the device as she will be relying on the design and analytical process to perform further testing. Finally the designers were established as the Major Qualifying Project (MQP) group: Brittany DiCapua, Meaghan Dunn, Tyler Modelski, and Kristin Sundberg. It was ultimately the designer's responsibility to translate the wants, needs and desires of all stakeholders into one device.

### 3.2 Clarification of Design Goals

To obtain a better understanding of the initial client statement the team analyzed the initial client statement provided by Professor Pins.

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*Build upon the current  $\mu$ DERM scaffold to enhance vascularization and the rate of tissue ingrowth, and create a method to assess and validate the efficiency of the design.*

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After further clarifying this client statement the team realized that this could be interpreted in many ways. After interviewing the client in multiple meetings, the team determined a list of objectives that would ultimately define the design of the project. Since the project had two goals, the first being enhancing vascularization and the second being validating the efficiency of the design, the team decided that two sets of objectives would best detail the client statement.

**Design Objectives for Enhancing Angiogenesis:**

- **Efficient**
- **Release Growth Factors at a Defined Rate**
- **Easy to Use**
- **Increases Tissue Ingrowth into Scaffold**

**Design Objectives for an Assay to Assess Efficiency of the Design:**

- **Efficient**
- **Quantifiable**
- **Effective**
- **Easy to Use**

One of the main concerns with the initial client statement was the particular system used to enhance the scaffold. Through developing the objectives above, the team determined that the most viable system would be the combination of a growth factor and controlled delivery method.

After the initial objectives of the design were established, other characteristics of the design were needed to solidify the boundaries and scope. Constraints are defined as characteristics of the design that need to be met under any circumstance or the device will fail. Constraints can be broken down into design constraints and project constraints. After researching and brainstorming, the team established the following lists of constraints for the project:

#### **Design Constraints:**

- **Maintain Structural Stability**
- **Collagen Sponge**
- **Biocompatible**
- **Cell Compatible**
- **Ability to be Sterilized**

#### **Project Constraints:**

- **Time (28 weeks)**
- **Budget (\$524 refundable)**
- **Material Access**
- **Lab Equipment Availability**

After interviewing the client, constraints of the design included utilization of a collagen sponge with the same structural stability as  $\mu$ DERM which is biocompatible, cell compatible, and able to be sterilized. It was determined that maintaining the collagen sponge is a design

constraint as the user and client do not want to change the core design. In most medical devices, biocompatibility is a constraint because if the materials used are not compatible with the body, they are unsuitable for medical applications. The design must be biocompatible and cell compatible to allow for culture of endothelial cells and fibroblasts. The design must also be able to be sterilized using the same method of sterilization for the current  $\mu$ DERM (incubation in antibiotics). The current collagen-GAG sponge has not been quantified for scaffold stability; however, studies on collagen-GAG sponges that are fabricated with 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) cross-linking (same method as the current) have been performed (Boyce et al., 2006).

Project constraints included time of completion, budget, material access, and lab equipment availability. The time constraint was very important in that there were only 28 weeks to complete the project. This prompted the need for a strict schedule throughout the year to keep the project and team on track for on time completion. The team also had a budget constraint of \$524.00 to be used on the design. It was thus crucial to find materials that were affordable or free through utilization of materials from labs or other resources on campus. Other project constraints were determined to be material access and lab equipment availability. There was the possibility that a necessary material was not available. There was also the possibility that a piece of equipment was not able to be located on campus. Both design and project constraints were important when ranking alternative design ideas.

### 3.2.1 Specifying Objectives

After establishing the initial objectives of both the design and the assay, the objectives were organized into primary objectives and secondary objectives. Primary objectives were marked with roman numerals (I, II, III...) and multiple secondary objectives were marked with

letters (a, b, c...) beneath their corresponding primary objective. This was used to specify each layer of the design's goals in an understandable manner.

### **Goal: Enhance Angiogenesis**

#### **I. Efficient**

- a) Cost effective (affordable for client)
- b) Minimal addition of complexity to current scaffold design
- c) Minimal manufacturing time of device
- d) Reproduced accurately between batches

#### **II. Release factors at defined rate**

- a) Release factors at constant rate
- b) Be able to precisely tailor the release rate
- c) Create scaffold with precise and reproducible release rate
- d) Create scaffold with accurate release rate

#### **IV. Easy to use**

- a) Minimal processing and handling time
  - i) Minimizes contamination
- b) Easy to fabricate
  - i) Time to fabricate
  - ii) Equipment used to fabricate
- c) Minimal complexity added to fabrication process
- d) Maintain sterilization method

#### **V. Increase Tissue Ingrowth to Scaffold**

- a) Increase endothelial cell migration into sponge *in vitro*

- b) Initiate sprouting of endothelial cells for potential blood vessel formation *in vivo*

### **Goal: Assay to Assess Efficiency of the Design**

#### **I. Efficient**

- a. High throughput
- b. Cost effective
- c. Minimize time for data collection
- d. Produces accurate results
- e. Produces precise/reproducible results

#### **II. Quantifiable**

- a. Quantifies EC migration into sponge
- b. Quantifies rate of release of growth factor (or protein of similar MW)
- c. Accurate quantification
- d. Precise/reproducible quantification

#### **III. Effective**

- a. Allows culture of ECs
- b. Allows for histological analysis
- c. Allows for fluorescence imaging
- d. Allows for staining of cells/proteins

#### **IV. Ease of Use**

- a. Easily assembled/prepared
- b. Minimal time for assembly
  - i. Reduce chance of contamination

c. Uses one variation of medium

As detailed above, each objective was broken down into more specific objectives to better understand the details that the design must have. A more thorough, detailed list of the objectives and sub-objectives with corresponding objectives trees can be found Appendix A. Also, explanations for each sets of objectives has been provided and can be found in Appendix B.

### 3.2.2 Quantitative Analysis of Objectives

The next step to the design process was to provide quantitative analysis of the seven primary design objectives. This analysis was used to determine the importance of each objective and to provide focus as to which objectives should be fulfilled first. To provide quantitative analysis, the team developed a pairwise comparison chart (PCC) to compare each of the primary objectives and sub-objectives for both the scaffold and assay. In the chart, each objective was compared against each other and given a score of 1, 0.5, or 0. The score corresponded to the objective that was in that row. A score of “1” indicated that the objective was more important than its comparison. A score of “0.5” indicated that the objective was equally as important as its comparison. A score of “0” indicated that the objective was not as important as its comparison. The PCC was filled out by the client, user, and design team to gather input from all views of the design. After the objectives and sub-objectives were ranked by each stakeholder, they were averaged and ranked based on importance. The charts below are a summary of Professor Pins (client), Amanda Clement (user), and the team (designers) rankings:

### Goal: Enhance angiogenesis

Table 1: Shows the pairwise comparison chart results from the client, user, and design team for the primary objectives of the goal to “enhance angiogenesis”.				
<i>Primary Objectives</i>	Professor Pins	Amanda Clement	Team	Average
Efficient	0	0	1.0	0.33
Release Factors at a Defined Rate	3.5	2.0	2.0	2.5
Easy to Use	1.0	1.0	0	0.67
Increase Tissue Ingrowth in Scaffold <i>in vitro</i>	3.0	4.0	3.5	3.5

As seen from Table 1, it was determined that the two most important primary objectives for the scaffold to enhance angiogenesis was to 1) increase tissue ingrowth into the scaffold *in vitro* and 2) to release growth factors at a defined rate. The two lesser important objectives include efficiency and ease of use. After ranking the primary objectives the team ranked the sub-objectives. These rankings can be found in Table 2.

Table 2: Shows the pairwise comparison chart results from the client, user, and design team for the secondary objectives of the objective “efficient”.				
<i>Primary Objectives</i>	Professor Pins	Amanda Clement	Team	Average
Cost Effective	0	0	0	0
Minimal Complexity of Current Design	1.5	1.0	1.5	1.3
Minimal Manufacturing	1.5	2.0	1.5	1.7
Reproduced Accurately Between Batches	3.0	3.0	3.0	3.0

It was determined that the most important sub-objective for efficiency is its reproducibility between fabrication of batches. This was important because the client and user would need a consistent model that could be tested- otherwise testing could not be considered significant because each fabricated design was not the same. It was also significant to note that cost effectiveness was ranked “0” for each stakeholder, which indicated that all were willing to



have a more expensive design if it produced the results needed. Table 3 shows the PCC results from the client, user, and team for the objective easy to use.

<b>Table 3: Shows the pairwise comparison chart results from the client, user, and design team for the secondary objectives of the objective “easy to use”.</b>				
<i>Easy to Use</i>	<b>Professor Pins</b>	<b>Amanda Clement</b>	<b>Team</b>	<b>Average</b>
<b>Minimal Processing and Handling Time</b>	0.50	1.0	0.50	0.67
<b>Easy to Fabricate</b>	0.5	1	0.5	0.67
<b>Minimal Complexity Added to Fabrication Process</b>	1.5	2.0	2.0	1.8
<b>Maintain Sterilization Methods</b>	X	X	X	X

The objective, easy to use, was of main importance to the user (Amanda), as she will ultimately be the one manipulating the final design. According to the PCC, adding minimal complexity to the fabrication process was the most important sub-objective. If the fabrication process became too complex, there was a chance the user would not be able to fabricate it consistently, which would have a large effect on the ease of fabrication, fabrication time, and reproducibility. It should also be noted that maintaining the sterilization method of the scaffold was not ranked in the PCC, due to the fact that it was determined to be a constraint of the design instead of a sub-objective. Table 4 shows the PCC results from the client, user, and team for the objective release factors at a defined rate.

<b>Table 4: Shows the pairwise comparison chart results from the client, user, and design team for the secondary objectives of the objective “release factors at defined rate”.</b>				
<b><i>Primary Objectives</i></b>	<b>Professor Pins</b>	<b>Amanda Clement</b>	<b>Team</b>	<b>Average</b>
<b>Release Factors at a Constant Rate</b>	3.0	0	2.0	1.7
<b>Precisely Tailor Release rate</b>	0	1.0	0	0.33
<b>Precise and Reproducible Release Rate</b>	1.5	3.0	2.0	2.2
<b>Accurate Release rate</b>	1.5	2.0	2.0	1.8

In order to best mimic the stages of wound healing, it was ideal for growth factors to be released at a defined rate. It was determined through the PCC for the objective, release factors at a defined rate, that the most important sub-objectives were having a precise, reproducible, and accurate release rate. These were most important because the design would ensure that the rate of release could easily be reproduced for the release mechanism of choice. The release method should also release growth factors at an accurate rate which mimics wound healing. Table 5 shows the PCC results from the client, user, and team for the objective increase tissue ingrowth to the scaffold.

<b>Table 5: Shows the pairwise comparison chart results from the client, user, and design team for the secondary objectives “increase tissue ingrowth to scaffold”.</b>				
<b><i>Primary Objectives</i></b>	<b>Professor Pins</b>	<b>Amanda Clement</b>	<b>Team</b>	<b>Average</b>
<b>Increase Endothelial Cell Migration into Sponge <i>in vitro</i></b>	1.0	1.0	1.0	1.0
<b>Initiate Sprouting of Cells for Blood Vessel Formation <i>in vivo</i></b>	0	0	0	0

One of the main strategies to promote vascularization was to increase tissue ingrowth into the scaffold. There were two main aspects to this strategy which included increasing endothelial cell migration into the collagen sponge and initiating sprouting for potential blood vessel

formation. Through the PCC, it was unanimous that the most important sub-objective was to increase endothelial cell migration. This narrowed the goal, enhancing vascularization, significantly as it focused the scope of the objective and the design as a whole.

### **Goal: Assay to Assess Efficiency of the Design**

Since there were two main goals of the project, as discussed in section 3.2, it was important to assess them separately. This section differs from the previous section as it outlines the PCC for the outline rather than the scaffold. Table 6 summarizes the PCC results of the client, user, and design team.

<b>Table 6: Shows the pairwise comparison chart results from the client, user, and design team for the primary objectives of the goal to create an assay to assess efficiency of the design.</b>				
<b><i>Primary Objectives</i></b>	<b>Professor Pins</b>	<b>Amanda Clement</b>	<b>Team</b>	<b>Average</b>
<b>Efficient</b>	1.0	0	1.0	0.67
<b>Quantifiable</b>	2.5	2.0	2.0	2.2
<b>Effective</b>	2.5	3.0	3.0	2.8
<b>Ease of Use</b>	0	1.0	0	0.33

After performing the PCC for the primary objectives for the assay, it was determined that the most important objectives were to create an effective and quantifiable assay. Effectiveness was important as the assay must be able to mimic the system required to validate the design objectives. In addition, it must be quantifiable so that there was solid data to provide proof of concept of the design.

<b>Table 7: Shows the pairwise comparison chart results from the client, user, and design team for the secondary objectives of the objective “efficient”.</b>				
<i>Primary Objectives</i>	<b>Professor Pins</b>	<b>Amanda Clement</b>	<b>Team</b>	<b>Average</b>
<b>High Throughput</b>	1.0	2.0	1.0	1.3
<b>Cost Effective</b>	0	0	1.0	0.33
<b>Minimize Data Collection Time</b>	2.0	1.0	1.0	1.3
<b>Produce Accurate Results</b>	3.5	4.0	3.5	3.7
<b>Produce Precise/ Reproducible Results</b>	3.5	3.0	3.5	3.3

The most important sub-objectives for the objective, efficient, were to produce accurate and reproducible results, as shown in Table 7. This indicated that the stakeholders believed the assay should allow for testing of the design to be accurate in comparison to the intended results of the scaffold design. It also indicated that the results should be reproducible between different trials of testing.

<b>Table 8: Shows the pairwise comparison chart results from the client, user, and design team for the secondary objectives of the objective “quantifiable”.</b>				
<i>Primary Objectives</i>	<b>Professor Pins</b>	<b>Amanda Clement</b>	<b>Team</b>	<b>Average</b>
<b>Quantifies Cell Migration to Sponge</b>	2.0	2.0	2.0	2.0
<b>Quantifies Rate of Growth Factor Release</b>	0	0	0	0
<b>Accurate Quantification</b>	2.0	2.5	2.0	2.2
<b>Produce Precise/ Reproducible Results</b>	2.0	1.5	2.0	1.8

The most important sub-objectives for the objective, quantifiable, were the ability of the assay to quantify endothelial cell migration into the sponge and be accurate, as seen in Table 8. This indicated that the assay would allow quantification of endothelial cell migration upwards into the sponge. Also, this method would be accurate and not subjective.

<b>Table 9: Shows the pairwise comparison chart results from the client, user, and design team for the secondary objectives of the objective “effective”.</b>				
<i>Primary Objectives</i>	<b>Professor Pins</b>	<b>Amanda Clement</b>	<b>Team</b>	<b>Average</b>
<b>Allows Cell Culture</b>	3.0	3.0	3.0	3.0
<b>Allows for Histological Analysis</b>	0	0	0.50	0.17
<b>Allows for Fluorescence Imaging</b>	2.0	2.0	2.0	2.0
<b>Allows for Cell/ Protein Staining</b>	1.0	1.0	0.50	0.83

The most important sub-objective for the objective, effective, was determined to be the allowance of endothelial cell culture, as seen in Table 9. This was very important, because if the assay did not allow for endothelial cell culture, there would be no way to assess the efficacy of the design. The assay also needed to allow for imaging so that quantification of endothelial cell migration could be obtained.

<b>Table 10: Shows the pairwise comparison chart results from the client, user, and design team for the secondary objectives of the objective “ease of use”.</b>				
<i>Primary Objectives</i>	<b>Professor Pins</b>	<b>Amanda Clement</b>	<b>Team</b>	<b>Average</b>
<b>Easily Assembled and Prepared</b>	2.0	1.0	0.50	1.2
<b>Minimal Time for Assembly</b>	1.0	2.0	0.50	1.2
<b>Uses One Variation of Medium</b>	0	0	2.0	0.67

For the primary objective, ease of use, there were two sub-objectives that were both ranked equally as important, as shown in Table 10. These sub-objectives were important in that the user would be able to easily assemble the assay through following a specific protocol. It would also require the minimum amount of assembly time which would help avoid potential problems such as contamination of endothelial cell cultures.

### 3.2.3 Revised Client Statement

Through analysis of objectives, sub-objectives, and further research, a revised client statement was formed from the original client statement: “Build upon the current  $\mu$ DERM scaffold to enhance vascularization and the rate of tissue ingrowth, and create a method to assess and validate the efficiency of the design.”

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***Modify the current  $\mu$ DERM scaffold by incorporating a growth factor and controlled release system into the porous collagen sponge. The factor should be released at a constant, tunable, precise and accurate rate and accelerate endothelial cell migration into the sponge. The design should also be easy to use, cost effective, efficient and add little complexity to current fabrication process. The design must also be validated using a cheap, high throughput assay. This assay should assess the rate of endothelial cell migration as well as rate of controlled release of the growth factor and should also allow for quantifiable results. The assay should also be easy to use and have reproducible, accurate, and precise results.***

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The two main objectives discussed in the revised client statement include the design of a controlled release system of a growth factor that would enhance angiogenesis, and the design of an assay that would assess migration of endothelial cell into the collagen sponge. These two goals of the project were detailed significantly based upon the objectives and sub-objectives ranked in the PCCs and incorporated into the revised statement.

### 3.3 Design Approach

#### 3.3.1 Management Approach

The team developed a management approach to help stay on track and meet specific requests. A Gantt Chart, found in Appendix C, was created as a timeline for specific deadlines and obligatory requirements. This chart allowed for scheduling of all assets of the project and timeline predictions. Throughout the duration of the project, each task was updated with a “percentage complete”, indicating which areas were falling behind track and should be focused on in more detail. The team continued to update the chart and track any advancements or drawbacks and reconfigure accordingly. Additionally, the team held weekly meetings with the client and user to ensure successful progression. In these meetings, presentations were given and built upon from week to week. Client and user meetings also ensured that the project remained focused and on the correct path for all stakeholders. Once a final design was chosen, the final schedule for ordering materials, fabricating scaffold parts, assembling the assay, and ultimately testing the design *in vitro* were developed in detail.

#### 3.3.2 Design Approach

##### 3.3.2.1 The Approach

In order to ensure a successful project, a technical approach was made to plan the design process. The first step to the technical approach was to use the objectives, constraints, and functions that had been defined for the design to develop possible design ideas and sketches. From there, three design alternatives were chosen and ranked based on how well they fit the most important objectives of the project. This allowed final design determination in a quantitative manner, providing solid proof of why it was chosen. The final design was then drawn in computer aided design (CAD) to the specific dimensions. Once the final design was

solidified, materials needed for its fabrication and testing mechanism were ordered. At this point, specific protocols for fabrication of the design as well as preparation of the testing assay were obtained and a schedule was made for assembly. First, the design was fabricated and its design components verified. From there, the assay for testing endothelial cell outgrowth was assembled. Once assembled, testing was performed followed by a quantitative analysis of the data. This led to modifications of the design to improve its efficacy and the possibility of further testing before a final product was presented.

#### *3.3.2.2 Technical Limitations*

In any design project, there are possible limitations or problems that could arise throughout the technical approach. A potential obstacle was the project budget and whether or not enough money was allotted to incorporate all ideas into the design. Another possible limitation was failure to fabricate the design from the fabrication process of choice. Some of the potential problems that could have been out of the team's hands were the cells adhering to the gel *in vitro* resulting in scarce cell migration. This could have happened if the cells were proliferating in their environment and attracted to the gel more so than the growth factors embedded into the scaffold. Also, with any cell culture experiment, the possibility of assay contamination could have been a major setback for the movement of the project. Meticulous labeling and cell culture techniques were essential in avoiding these limitations.

#### *3.3.3 Financial Approach*

As outlined in the constraints for the project, the team was allotted \$524.00 to be reimbursed by Worcester Polytechnic Institute (WPI) for the design. After preliminary research, the team found that commercial prices for growth factors, materials, cells, and medium were



very expensive. Although purchasing some of these materials, the team used outside resources and connections to obtain other necessary materials.

## 4.0 Alternative Designs

### 4.1 Needs Analysis

In order to better identify the needs and wants of the system, the team performed a systemic needs analysis for both the scaffold and the assay. Systemic needs for the scaffold and assay are defined as metrics that must be maintained to ensure necessary objectives are met in order to warrant an effective design. Systemic wants for the scaffold and assay were desirable, preferred metrics that were not detrimental to the efficacy of the design. Systemic needs analysis charts for both the scaffold and assay are shown below. Table 11 represents the needs for the scaffold design, and Table 12 the wants for the scaffold design. Similarly, Table 13 represents the needs for the experimental assay and Table 14 the wants of the experimental assay.

<b>Table 11: Scaffold needs and corresponding definitions.</b>	
<b>Scaffold Needs</b>	<b>Definition</b>
Increase migration	Ability of the scaffold to enhance tissue ingrowth via endothelial cell migration to the collagen sponge <i>in vitro</i>
Maintain porosity	Ability of the designer to maintain scaffold stability <i>in vitro</i> by controlling the porosity of the collagen sponge
Maintain degradation properties	Ability of the designer to maintain scaffold stability <i>in vitro</i> through controlled degradation properties
Flexibility	Ability of the scaffold to maintain <i>stability in vitro</i> through minimal collagen sponge contraction
Precise and reproducible release rate	Ability of the scaffold to release growth <i>factors in vitro</i> at a defined rate that is consistent between batches
Accurate release rate	Ability of the scaffold to release growth <i>factors in vitro</i> at a defined rate that is consistent with valid scientific research

Accurate reproducibility	Ability of the designer to produce the scaffold multiple times with consistent results between batches
Minimal contraction	Ability of the scaffold to maintain stability <i>in vitro</i> through controlled vertical flexibility

**Table 12: Scaffold wants and corresponding definitions.**

<b>Scaffold Wants</b>	<b>Definition</b>
Increase sprouting	Ability of the scaffold to enhance tissue ingrowth via endothelial cell sprouting <i>in vitro</i>
Tailored release rate	Ability of the designer to alter the release rate of growth factors <i>in vitro</i> to desired specifications
Minimal complexity	Ability of the scaffold to be produced through a process with as few components as possible
Ease of fabrication	Ability of the designer to produce the scaffold with few to no complications
Minimal processing	Ability of the scaffold to be produced with as minimal processing requirements as possible
Cost effective	Ability of the scaffold results to outweigh its production cost

**Table 13: Assay needs and corresponding definitions.**

<b>Assay Needs</b>	<b>Definition</b>
Endothelial culture	Ability of the assay to allow for simultaneous culture of the endothelial cells
Accurate quantification	Ability of the assay to numerically assess the results so that data can be easily interpreted and analyzed
Quantify migration	Ability of the assay to numerically assess the vertical movement of endothelial cells toward and into the collagen sponge
Precise and reproducible quantification	Ability of the assay to yield consistent data between trials
Quantify growth factor release	Ability of the assay to numerically assess the rate at which the growth factor is releasing
Reasonably accurate results	Ability of the assay to yield data that is reasonably consistent with the scientific literature

Table 14: Assay wants and corresponding definitions.	
Assay Wants	Definition
Imaging	Ability of the assay to assess results through the use of cellular imaging
Cellular staining	Ability of the assay to assess results through the use of cellular staining
High through-put	Ability of the assay to analyze a variety of results in a time-efficient manner with consistent data
Cost effective	Ability of the assay results to outweigh the production costs
Minimal time in data collection	Ability of the assay to allow for assessment of results in the shortest amount of time possible
Reproducible	Ability of the designer to produce the assay multiple times with consistent results between trials
Ease of assembly	Ability of the designer to produce the assay with as few complications as possible
Cellular medium	Ability of the assay to only require one type of cellular medium for culture

#### 4.1.1. Systemic Needs

##### 4.1.1.1. Systemic Needs for Scaffold

Systemic needs for the scaffold design were determined by consideration of the highest ranked objectives seen in the pairwise comparison charts. The team brainstormed and categorized the objectives as crucial metrics that warrant an effective design. The consequent placement of each systemic need was discussed with the client. It was determined that the scaffold must enhance tissue ingrowth via endothelial cell migration into the collagen sponge *in vitro*. The designer must be able to maintain scaffold stability *in vitro* by controlling the porosity of the collagen sponge and the degradation properties of the scaffold. The scaffold must maintain its stability *in vitro* through minimal collagen sponge contraction and controlled vertical flexibility. It must release growth factors *in vitro* at a defined rate that is consistent between batches and consistent with valid scientific research. The designer must be able to produce the scaffold multiple times with consistent results between batches.

#### 4.1.1.2. Systemic Needs for Assay

Systemic needs for the experimental assay were determined in the same manner as systemic needs for the scaffold. The team brainstormed and categorized the objectives as crucial metrics that warrant an effective design. The consequent placement of each systemic need was discussed with the client. It was determined that the experimental assay must allow for simultaneous culture of endothelial cells to ensure non-destructive data and accurate quantification. Vertical migration of endothelial cells in culture must be numerically defined and quantified to demonstrate the efficacy of the scaffold design in enhancing angiogenesis *in vitro*. The release of growth factor must also be numerically defined in the experimental assay to properly assess the effect of the factor on endothelial cell migration and further, angiogenesis. Lastly, data obtained in each experimental trial must be accurate and must not deviate from expected numeric found in literature.

#### 4.1.2 Systemic Wants

##### 4.1.2.1. Systemic Wants for Scaffold

Systemic wants for the scaffold were determined in the same manner as systemic needs. Highly ranked objectives from the pairwise comparison charts were discussed by the team and client, and categorized accordingly. The team defined systemic wants as metrics that were important and not detrimental to the efficacy of the design. It was therefore determined that the scaffold should enhance tissue ingrowth via endothelial cell sprouting *in vitro*. The designer should be able to alter the release rate of growth factors *in vitro* to desired specifications. The scaffold should be produced through a process with as few components as possible and the designer should therefore be able to produce the scaffold with few to no complications. The

scaffold should be produced with as minimal processing requirements as possible, and ultimately the scaffold's results should outweigh its production cost in order to ensure cost effectiveness.

#### 4.1.2.2. Systemic Wants for Assay

Systemic wants for the experimental assay were determined in the same manner as systemic needs. Highly ranked objectives from pairwise comparison charts were discussed by the team and client, and categorized accordingly. Systemic wants are important metrics which were not detrimental to the efficacy of the design. It was determined that the experimental assay should quantify endothelial cell migration through fluorescent cellular staining and imaging. Imaging endothelial cells will allow for accurate quantification of cellular movement through visual aid. The assay should possess the ability to analyze a variety of results in a time efficient manner and be easily reproduced and assembled between trials. Lastly, the experimental assay should be a cost effective way to assess endothelial cell migration and further angiogenesis *in vitro*.

## 4.2 Functions and Specifications

Using the two highest ranked objectives from the PCCs, the team established specific functions for 1) the scaffold and 2) the experimental assay.

### 4.2.1 Functions for the Scaffold Design

The highest ranked objective for the scaffold design was to increase tissue ingrowth into the collagen sponge. To do so, the scaffold design must allow for an *increase in endothelial cell migration*. *Endothelial cell viability* must be maintained in order to ensure acquisition of quantifiable results and ultimately a successful system. Lastly, to promote adequate tissue ingrowth *in vivo*, the scaffold design *must mimic the process of angiogenesis in vitro*. The second highest ranked objective for the scaffold design was to release factors at a defined rate. It is

imperative that the chosen factor *provides a controlled release*. A controlled release rate will allow for a defined concentration of factor release. Table 15 shows each function for the two highest ranked objectives for the scaffold

<b>Table 15: Two highest ranked objectives and corresponding functions for the scaffold.</b>	
<i>Scaffold</i>	
<b>Increase Tissue Ingrowth into Collagen Sponge</b>	Increase endothelial cell migration
	Maintain endothelial cell viability
	Enhance angiogenesis
<b>Release Factors at a Defined Rate</b>	Provide controlled release

#### 4.2.2 Functions for the Assay Design

The highest ranked objective for the experimental assay was the ability of the assay to be effective. To ensure an effective assay, the design must *allow for endothelial cell culture*. If the assay does not allow for continuous culture of endothelial cells, the cells may be destroyed. This will inhibit adequate analysis and consequentially interferes with evidence that proves desired objectives were satisfied. Additionally, the assay must *allow for cellular marking*. If the cells are not marked or labeled, cellular movement and morphology will be difficult to analyze and quantify, thus diminishing the effectiveness of the assay. The second highest ranked objective for the experimental assay was to produce results that are quantifiable; therefore, it must *allow for accurate quantification of data*. Additionally, the assay must *allow for non-destructive data collection*. A destructive assay may affect the project budget and be detrimental to the experimental procedure thus hindering desired results. Table 16 below shows each function for the two highest ranked objectives for the experimental assay.

<b>Table 16: Two highest ranked objectives and corresponding functions for the assay.</b>	
<i>Experimental Assay</i>	
<b>Effective</b>	Allow for endothelial cell culture
	Allow for cellular marking
<b>Quantifiable</b>	Allow for quantification of data
	Allow for non-destructive data collection

#### 4.2.3 Specifications

Through utilization of each function stated above, the team established numerical specifications for the scaffold design. Design specifications provide quantitative information about the necessary requirements for a device in regards to device assembly and usage. The team established each design specification through ample literature and client consent. The collagen sponge used for this design was 336  $\mu\text{m}$  thick and two centimeters in diameter (Clement et al., 2013). To provide mechanical structure to the scaffold system, the device must maintain a porosity of 400  $\mu\text{m}^2$  and a stiffness of 35 mN/mm (Powell et al., 2006). If the scaffold is cross-linked with 5 mM EDC, it is expected that approximately 40% of the scaffold will degrade after 5 days; this is done with 60 mM EDC, there will be no predicted degradation (Powell et al., 2006). In order to ensure the scaffold device promotes tissue ingrowth and mimics the angiogenesis stage of wound healing, it is expected that factors will be released at a controlled rate over a four day time span. This will ensure proper migration of endothelial cells *in vitro* which will translate to improved vascularization *in vivo* (Singer et al., 1999).

#### 4.2.4 Functions-Means Brainstorming

Using the established design functions, the team conducted a brainstorming session to generate ideas for different means which could satisfy and fulfill each function for the scaffold design. Each team member was to research specific growth factors, conjugate methods or binders that could enhance angiogenesis and vascularization, and explain the pros and cons to each. Each design component was recorded on a white board and discussed as a team. Below, Figure 3 is a picture of the whiteboard where the team recorded potential design components for the scaffold. A second brainstorming session was held for the experimental assay. With consideration of the

design objectives, the team created numerous assay designs which would satisfy each function.

Preliminary sketches for the assay designs are shown below as Figure 4.

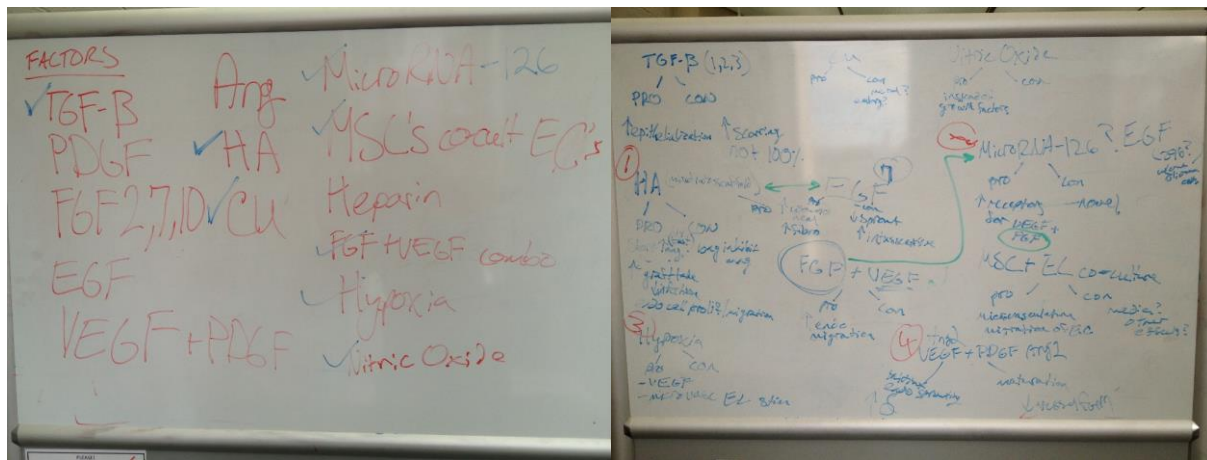


Figure 3: Picture of the white board used by the team to brainstorm specific growth factor components for the scaffold to be used to enhance tissue ingrowth and vascularization. After brainstorming, the team determined the top factors to improve vascularization include FGF-2, TGF-beta, MSC's, VEGF, and hyaluronic acid. These factors were specifically chosen for their reliability in the field as well as for their alignment with the client statement.



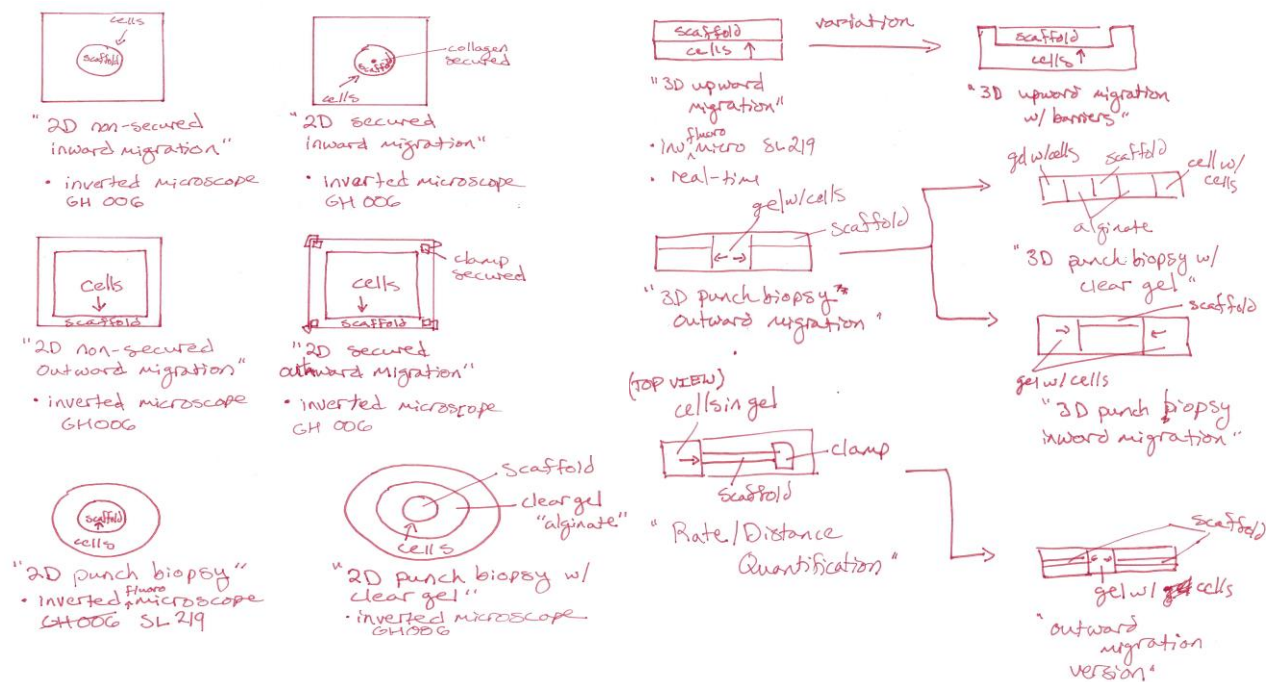


Figure 4: Preliminary sketches which the team conducted to explore different components for the experimental assay which would test the efficacy of the newly modified and enhanced design. The sketches include two dimensional and three dimensional migration assays.

Through the team brainstorming sessions, the team established means to achieve the stated functions for the scaffold design and assay. Given the time constraint for the project, the team and client established functions for only the two highest ranked objectives for the scaffold and assay. Due to time constraints and the high importance of these objectives in relation to the client statement, the team primarily focused on the top two objectives. Table 17 and Table 18 are representative of the established means from these corresponding functions for the scaffold and assay.

Table 17: Established means from the corresponding function for the two highest ranked objectives for the scaffold design	
Scaffold Objective: Increase Tissue Ingrowth into Sponge	
Function	Established Means
Increase endothelial cell migration	RGD Peptide
	VEGF
	Micro-Topography
	FGF-2

	Angiopoietin-1
<b>Maintain endothelial cell viability</b>	MSC's + EC's
	Hyaluronic Acid
	Heparin
	Mechanical Stretching
	5 mM EDC Crosslinking
	60 mM EDC Crosslinking
<b>Mimics angiogenesis</b>	Hypoxia
	Nitric Oxide
	FGF-2
	VEGF
	PDGF
	TGF-beta
<b>Provide controlled release</b>	EGF
	Gelatin microspheres
	PLGA Microspheres
	Binding Factor to Sponge

<b>Table 18: Established means from the corresponding function for the two highest ranked objectives for the experimental assay.</b>	
Assay Objective: Effective	
<b>Function</b>	<b>Established Means</b>
<b>Allow for endothelial cell culture</b>	Collagen
	Alginate
	PDMS
	Chitosan
	Agarose
<b>Allow for cellular marking</b>	GFP Marked Endothelial Cells
	Hoechst Staining
	Alexa-Flour488 Staining
<b>Allow for quantification of data</b>	<b>Manual Counting</b>
	MATLAB Programming
	ImageJ: Distance
	Cell Profiler
	ImageJ: Fluorescence
<b>Allow for non-destructive data collection</b>	Topographical Inward Migration
	Topographical Outward Migration
	Gel Inward Migration
	Gel Outward Migration
	Topographical Outward Boundary Migration
	Topographical Inward Boundary Migration
	Gel Inward Boundary Migration
	Gel Outward Boundary Migration

#### 4.2.5 Design Matrices Analysis

In order to determine design alternatives, a design matrix analysis of the potential means brainstormed for each objective-driven function was used. This allowed for quantified rankings of each mean to express the method for choosing each alternative design. The design matrix took each mean under each function and “ranked” each mean a score of 1-4 (4 being the most suitable and 1 not suitable) against each objective. In order to rank each mean 1-4, scoring criteria was established and can be seen in Appendix D. Scoring criteria was based off what was seen to be considered successful in literature or off the educated opinion of the team about how many fabrication steps, fabrication time, etc. would be ideal. After a mean was ranked 1-4 against each objective that correlated to it, the resulting score was multiplied by a weight factor. Weight factors for each objective were determined based of the PCCs presented in section 3.2.2. For example, the highest ranked objective based on the PCC was “increase tissue ingrowth into the scaffold *in vitro*”. Thus, in this example, its weighted multiplier was “4” and the lowest ranked objective was “efficient” was weighted at a multiplier of “1”. The weighting system produced a weighted value for each objective that the mean was compared to. This number was then totaled and normalized using the following equation:

$$\text{Normalized Score} = \text{Mean's Weighted Score} / \text{Maximum Weighted Total Score Possible}$$

This allowed for all means to be analyzed on a 100 point scale- 100 being the most suitable (all rankings of “4”) and 25 being the least suitable (all rankings of “1”). This design matrix was used for both the scaffold and assay and can be viewed in Appendix E. The results of ranking each mean against each other for each function can be seen in Tables 19 and 20.

<b>Table 19: Design matrix results for the scaffold design. 100% indicated the most suitable design and 25% indicated the least suitable design.</b>		
<b><i>Increase Endothelial Cell Migration</i></b>		
<b>1<sup>st</sup></b>	FGF-2	88%
<b>2<sup>nd</sup></b>	VEGF	84%
<b>3<sup>rd</sup></b>	Micro-Topography	72%
<b><i>Maintain Cell Viability</i></b>		
<b>1<sup>st</sup></b>	5 mM EDC Cross-linked	76%
<b>2<sup>nd</sup></b>	Heparin	69%
<b><i>Mimic Angiogenesis</i></b>		
<b>1<sup>st</sup></b>	FGF-2	83%
<b>2<sup>nd</sup></b>	VEGF	78%
<b>3<sup>rd</sup></b>	PDGF	64 %
<b><i>Controlled Release Rate</i></b>		
<b>1<sup>st</sup></b>	Gelatin Microspheres	87%
<b>2<sup>nd</sup></b>	Bind factor to Sponge	73%
<b>3<sup>rd</sup></b>	PLGA Microspheres	63%

<b>Table 20: Design matrix results for the assay design. 100% indicated the most suitable design and 25% indicated the least suitable design.</b>		
<b><i>Allow for Endothelial Cell Culture</i></b>		
<b>1<sup>st</sup></b>	Alginate	92%
<b>2<sup>nd</sup></b>	Collagen	87%
<b>3<sup>rd</sup></b>	Agarose	81%
<b><i>Allow for Non-Destructive Data Collection</i></b>		
<b>1<sup>st</sup></b>	Topographical Inward Migration	88 %
<b>2<sup>nd</sup></b>	Uni-Directional Single Strip	87%
<b>3<sup>rd</sup></b>	Topographical Outward Migration	86 %
<b><i>Allow for Quantification of data</i></b>		
<b>1<sup>st</sup></b>	MATLAB	89 %
<b>2<sup>nd</sup></b>	ImageJ	80 %
<b>2<sup>nd</sup></b>	Cell Profiler	80%
<b>2<sup>nd</sup></b>	ImageJ Fluorescence	80%
<b><i>Allow for Cellular Marking</i></b>		
<b>1<sup>st</sup></b>	GFP Marked Endothelial Cells	93%
<b>2<sup>nd</sup></b>	Alexa-Flouro488 Stain	89%
<b>3<sup>rd</sup></b>	Hoechst Stain	89%

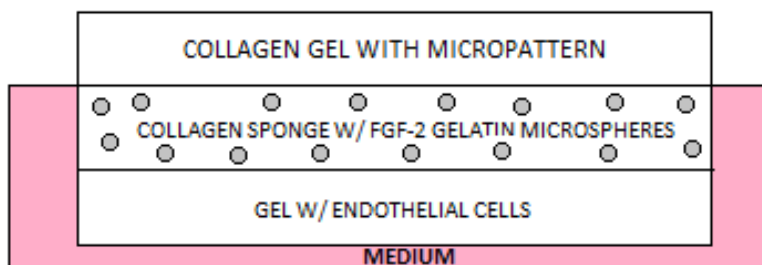
As seen from Tables 19 and 20, the number one factor to both enhance endothelial cell migration and angiogenesis was FGF-2. Both FGF-2 and VEGF were found in literature to be significantly more effective in enhancing endothelial cell migration compared to other factors; however, VEGF was much more expensive and a small portion of FGF-2 was available for the

team. Ideally, to maintain endothelial cell viability it was determined that 5 mM EDC cross-linking of a collagen-GAG sponge was ideal (Powell et al., 2006). From Table 19, it was determined that to prevent denaturation of FGF-2, heparin could be a good binding agent to incorporate into the scaffold (Ho et al., 2009). Controlled release rates of the factor was determined to be very suitable in gelatin microspheres as they showed tailorable release kinetics in literature and were readily available and free to manufacture (Kawai et al., 2005).

In determining the gel to be used for endothelial cell culture, alginate, collagen, and agarose gels all scored relatively high and were all available for free in the lab. Very similar results were seen for the allowance of non-destructive data collection. Many designs had been brainstormed as previously stated and further feasibility testing was performed on the highest scoring designs. For quantification of data, it was determined that the team ideally would use a MATLAB program to quantify migration due to the fact it is automated and accurate compared to manual data collection. The three means that fell in second place could all be used and their results compared to determine the most accurate and efficient method during the final assay. For cellular marking, the team ranked the top three means, but similarly to the above methods, will be evaluated during feasibility testing. The ranked results of both the scaffold and assay design matrices were factors which led to the formation of three design alternatives that are outlined in section 4.3.

## 4.3 Design Alternatives

### 4.3.1 Design Alternative 1: FGF-2 Impregnated Gelatin Microspheres Analyzed by a Topographical Inward Endothelial Cell Migration Assay

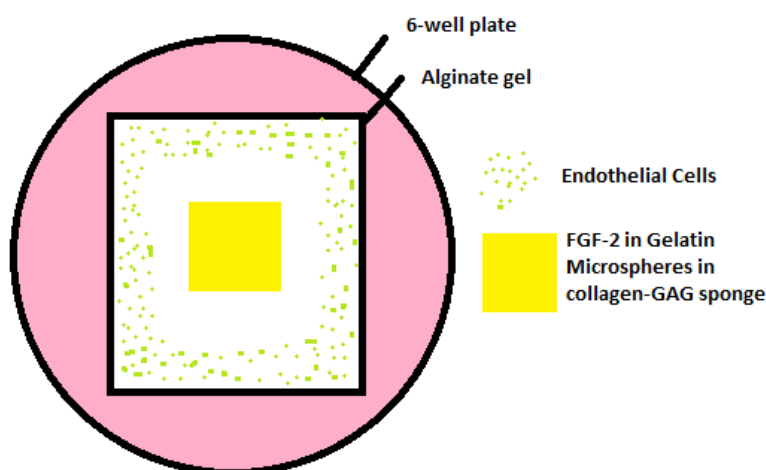


*Figure 5: Computer drawing of design alternative one. FGF-2 will impregnate dry gelatin microspheres which are injected into the collagen sponge.*

After analyzing the design matrices for the scaffold, design alternatives were made by combining the top scoring means for separate functional requirements. The number one design was determined to be the incorporation of FGF-2 impregnated gelatin microspheres within the collagen-GAG sponge. Figure 5 is a schematic of design alternative one. FGF-2 has been proven to enhance angiogenesis through fibroblast proliferation and in particular endothelial cell migration (Kawai et al., 2005). Gelatin microspheres would be used as a controlled release mechanism and could be tailored to the desired release rate by controlling microsphere diameter. Gelatin microspheres could retain FGF-2 through adsorption of the protein as the resultant of the electrostatic interactions of the positively charged FGF-2 and negatively charged gelatin (Tabata et al., 1998). Ideally, these could be injected into the pores of the collagen-GAG sponge. Table 21 shows a summary of the pros and cons of using FGF-2 impregnated microspheres.

<b>Table 21: Pros and cons list for the FGF-2 impregnated gelatin microspheres incorporated into the collagen-GAG sponge</b>	
<i>Fibroblast Growth Factor-2 + Gelatin Microspheres</i>	
<i>Pros:</i>	<i>Cons:</i>
Stimulates EC Migration	Cannot be used <i>in vivo</i> in free form due to short half-life
Increases synthesis of collagenase	Need balance of isoforms (2,7,10) for proper vascularization
Can be loaded in microspheres for effective delivery	FGF-2 is somewhat expensive
Can be combined because of suitable isoelectric points	Can't cross-link after protein is present to avoid denaturation
Range of biodegradability into non-toxic byproducts	
Injectable	
Gelatin microspheres inexpensive fabrication process	

The first design alternative for the assay included endothelial cells plated on an alginate gel with topographical inward endothelial cell migration. Figure 6 below shows the conceptual idea behind topographical inward endothelial cell migration.

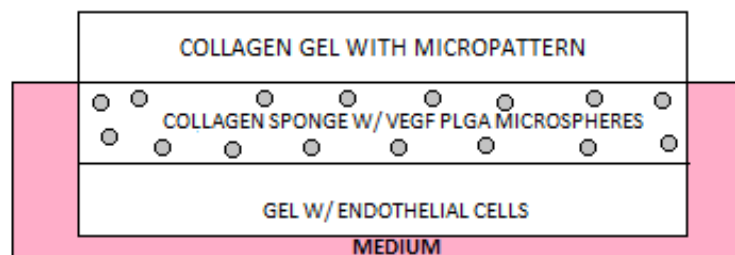


*Figure 6: Computer drawing of the topographical inward migration assay. The green represents seeded endothelial cells on an alginate gel and the yellow represents the scaffold and factor secured to the alginate gel. Migration of the cells inwards from all directions towards the growth factor/collagen sponge conjugate will be measured.*

As shown above, fluorescently labeled endothelial cells could be plated on the outer edges of an alginate gel with the angiogenic factor and sponge secured to the middle of the gel. The gel and sponge assembly could be placed in a six well plate and be completely engulfed in medium. Endothelial cell migration could be analyzed by measuring the gap closure between the cells and scaffold on multiple points on the gel and migration could be quantified by measuring the distance particular cells moved at multiple preceding time points after seeding. The pros and cons of this assay design are outlined in Table 22.

<b>Table 22: Pros and cons list for the topographical inward migration assay on an alginate gel.</b>	
<i>Topographical Inward Migration on an Alginate Gel</i>	
<b>Pros:</b>	<b>Cons:</b>
Simple and cheap fabrication	Migration may be hard to quantify precisely
Alginate supports EC viability	EC have ability to migrate diagonally
Non-destructive and allows for many data points	Release gradient of FGF-2 due to micro-fluidics
Gap closure imaging is clearer than imaging in scaffold	

#### 4.3.2 Design Alternative 2: VEGF Impregnated PLGA Microspheres Analyzed by a Unidirectional Single Strip Endothelial Cell Migration Assay



*Figure 7: Computer drawing of design alternative two. VEGF loaded PLGA microspheres were embedded into the collagen-GAG sponge.*

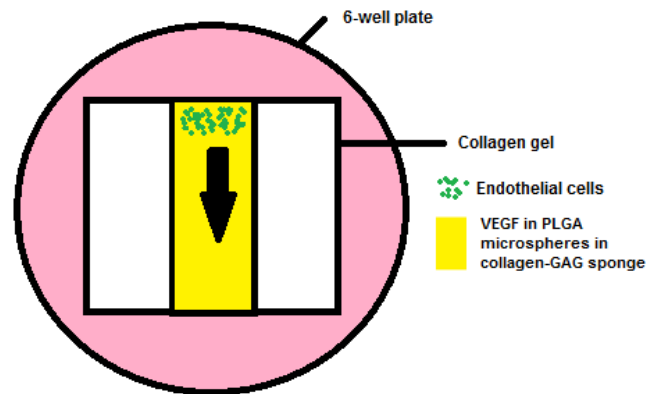


The second design alternative incorporated VEGF into PLGA microspheres within the collagen-GAG sponge. The apparatus was cross-linked using 5 mM of EDC. Figure 7 is a drawing of the proposed apparatus. VEGF is a well-known growth factor which has been proven to enhance angiogenesis and increase blood vessel formation *in vivo* (des Rieux et al., 2011). PLGA microspheres could be used as a mechanism for controlled release. It has been demonstrated by Liu et al. that PLGA microspheres produce porous structures with high drug loading and encapsulation capabilities (Liu et al., 2010). This could allow for a controlled, slowed, and sustained release of the growth factor. VEGF can be incorporated into PLGA microspheres through conjugation methods and protein adsorption. Table 23 is a summary of the pros and cons for using PLGA microspheres to deliver VEGF in the collagen-GAG sponge.

<b>Table 23: Pros and cons list for utilization of VEGF impregnated PLGA microspheres incorporated into the collagen-GAG sponge.</b>	
<i>Vascular Endothelial Growth Factor + PLGA Microspheres</i>	
<i>Pros:</i>	<i>Cons:</i>
VEGF induces neovascularization	PLGA potential for being toxic (synthetic)
PLGA has a highly tailorable release rate	Overexpression of VEGF can promote disease formation
VEGF can be effectively loaded into PLGA microspheres	VEGF is expensive
VEGF induces proliferation and migration of endothelial cells	VEGF has a short biological half-life

The second design alternative for the assay incorporated endothelial cells plated on the collagen-GAG sponge with examination of unidirectional migration. Figure 8 demonstrates the conceptual idea of the assay. A collagen gel could be added to a cell plate, and a strip of the collagen scaffold could be layered on top of the gel. Endothelial cells, stained with AlexaFluoro488 red to enhance cellular visibility, could be plated on the top of the scaffold strip. The migration of endothelial cells downward to the bottom of the scaffold strip could be

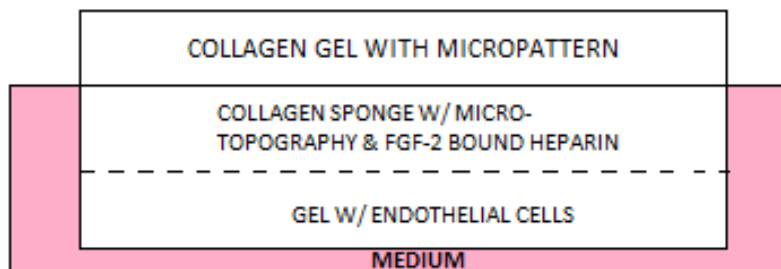
examined and quantified using an imaging processing program, ImageJ. The pros and cons of using the unidirectional single strip migration assay on the collagen gel are outlined below in Table 24.



*Figure 8: Computer drawing of the unidirectional single strip migration assay. The green represents the endothelial cells. The yellow represents the collagen-GAG scaffold with the incorporation of VEGF impregnated PLGA microspheres. Migration of the cells down the scaffold will be measured.*

Table 24: Pros and cons of using a unidirectional single strip migration assay on collagen gel.	
Unidirectional Single Strip Migration on Collagen Gel	
<i>Pros:</i>	<i>Cons:</i>
Migration analysis is cheap and easy to perform	User variability in migration measurements
Collagen will support endothelial cell viability	Endothelial cells may migrate diagonally
Non-destructive and allows for many data points	Migration may be hard to quantify precisely

#### 4.3.3 Design Alternative 3: Micro-topography Collagen-GAG Sponge with Heparin Bound to FGF-2 Analyzed by a Topographical Outward Endothelial Cell Migration Assay



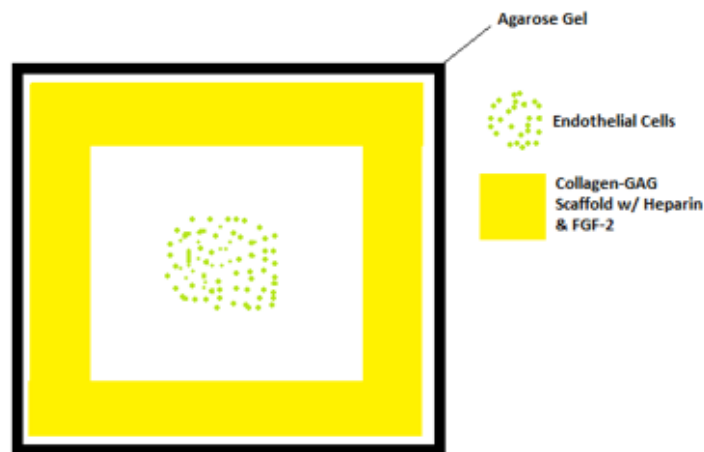
*Figure 9: Computer drawing of design alternative three. The heparin-bound micro-patterned collagen sponge will be combined with FGF-2.*

The third design alternative was the incorporation of FGF-2 into the collagen-GAG sponge through a heparin-bound micro-patterned surface. Heparin is a highly negatively-charged molecule known to specifically bind to growth factors and regulate their activity; FGF-2 displays a particularly positive surface charge that is complimentary to this anionic characteristic (Taylor, 2007). Micro-patterned surfaces have been shown to improve cell attachment and migration into scaffolds, and thus ultimately improve tissue formation (Papenburg et al., 2007). Heparin could be incorporated into the collagen sponge in two ways: during material synthesis or through postmodification of the prepared scaffold. To proceed with the former, heparin could be added to the collagen solution prior to fabrication and stabilization. To proceed with the latter, heparin could be dissolved in a buffer into which the crosslinked scaffold could be added. This would be followed by rinses to remove unbound heparin, and finally freeze-drying (Knaack et al., 2013). The collagen sponge could be either cast onto a pattern with the incorporation of heparin (method one) or prior to submersion in the heparin buffer. The addition of FGF-2 to the scaffold

could be the final step necessary to complete design alternative three. Figure 9 depicts a drawing of the apparatus, and Table 25 below displays the pros and cons of this option.

<b>Table 25: Pros and cons list for heparin-bound micro-patterned collagen sponge with addition of FGF-2.</b>	
<b><i>Heparin-bound Micro-patterned Sponge + Fibroblast Growth Factor-2</i></b>	
<b><i>Pros:</i></b>	<b><i>Cons:</i></b>
Stimulates growth of fibroblasts and EC Migration	Cannot be used <i>in vivo</i> in free form due to short half-life
Increases synthesis of collagenase	Need balance of isoforms (2,7,10) for proper vascularization
Heparin has affinity for FGF2	FGF-2 is somewhat expensive
Heparin binding to FGF2 increases half life	Doesn't extend FGF2 release rate
Extremely low isoelectric point for protein binding	Possibility of mechanical failure
Microtopography increases surface area	Fabrication challenges (ability to change sponge topography)
Microtopography shown to attract EC migration	

The third design alternative for the assay included endothelial cells plated on an agarose gel with topographical outward endothelial cell migration. Figure 10 shows the conceptual idea behind topographical outward endothelial cell migration.



*Figure 10: Computer drawing of the topographical outward migration assay. The green represents seeded endothelial cells on an agarose gel and the yellow represents the scaffold and factor secured to the gel. This representation does not depict the surrounding culture plate. Cell migration will be examined outward in all directions towards the collagen sponge FGF-2 heparin conjugate boarder.*

As shown, the collagen-GAG scaffold (with microtopography, heparin, and FGF2) could surround a bolus of fluorescently stained endothelial cells. A uniform size square could be cut from the center of the scaffold with a razor blade to allow for injection of the cells. The center cut-out could not span the width of the agarose gel on the bottom of the plate so that cells could still be in contact with it. The gel and sponge assembly could be placed in a six-well plate and completely submerged in medium to sustain cell viability. Migration could be observed and analyzed by examining the gap closure between the cells and scaffold at multiple points on the gel. It could be quantified by measuring the distance that particular cells moved at various time points after seeding. The pros and cons of this assay are shown in Table 26

<b>Table 26: Pros and cons list for the topographical outward migration assay on an alginate gel.</b>	
<i>Topographical Outward Migration on an Agarose Gel</i>	
<i>Pros:</i>	<i>Cons:</i>
Simple and cheap fabrication	Migration may be hard to quantify precisely
Agarose supports EC viability	EC have ability to migrate diagonally
Non-destructive and allows for many data points	Release gradient of FGF-2 may be affected by micro-fluidics
Gap closure imaging is clearer than imaging in scaffold	

## 5.0 Feasibility Study/Experiments

In some cases, additional research is sufficient to quantify one mean over the other, but when the top three means are ranked closely and there is not ample research to differentiate them, experiments are completed to further determine the components for the final design.

### 5.1 Cell Culture

#### 5.1.1 Material for Cell Culture

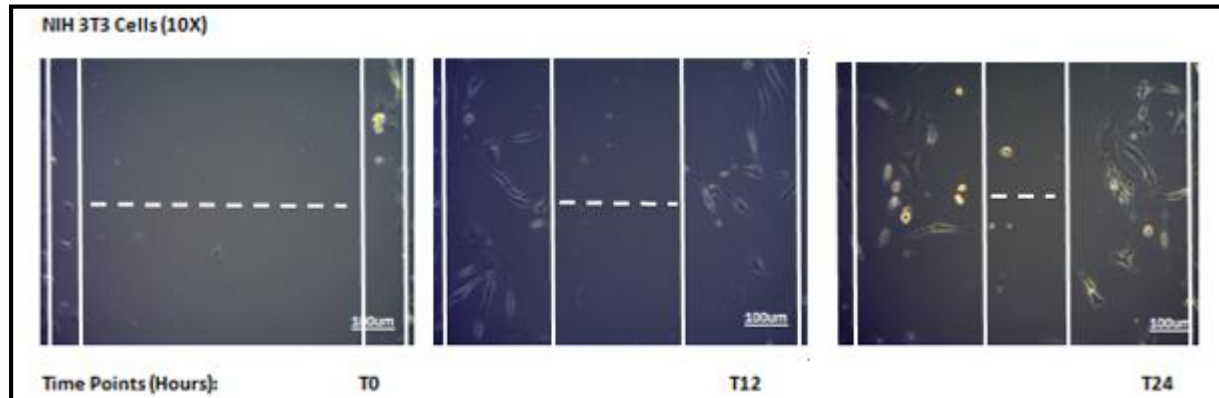
##### *5.1.1.1 Collagen versus Agarose and Alginate*

Alginate, agarose, and collagen gels were examined in the laboratory and their fabrication and imaging abilities were assessed. Fabrication methods for collagen, agarose, and alginate gels can be seen in Appendix F. Collagen was clear and weak in mechanical stability. Alginate and agarose were both thick and translucent. Alginate, like collagen, had poor mechanical stability, whereas agarose was stronger and not as easily broken. Due to the mechanical integrity of agarose, it was able to be cut into thin slices. These small marks caused light diffraction and contributed to high background light in microscope images, making it harder to fabricate and creating a rough surface that was poor for imaging. A second fabrication limitation of alginate was that it was not possible to make a flat gel. NIH/3T3 fibroblasts were seeded onto each gel

type and their ability to be imaged was assessed. The images can be seen in Appendix G. The collagen gel was found to yield the most optimal images.

#### 5.1.1.3 Outgrowth Assay

The team ran an outgrowth assay to determine if NIH 3T3 cells migrated on polystyrene. Cells were plated on a polystyrene dish and allowed to come to ~70% confluence. A scratch was made using a plastic pipette tip. Movement from the edges of the scratch towards the middle was monitored, with pictures taken at 0, 12, and 24 hours. Figure 11 details the results of the outgrowth assay.



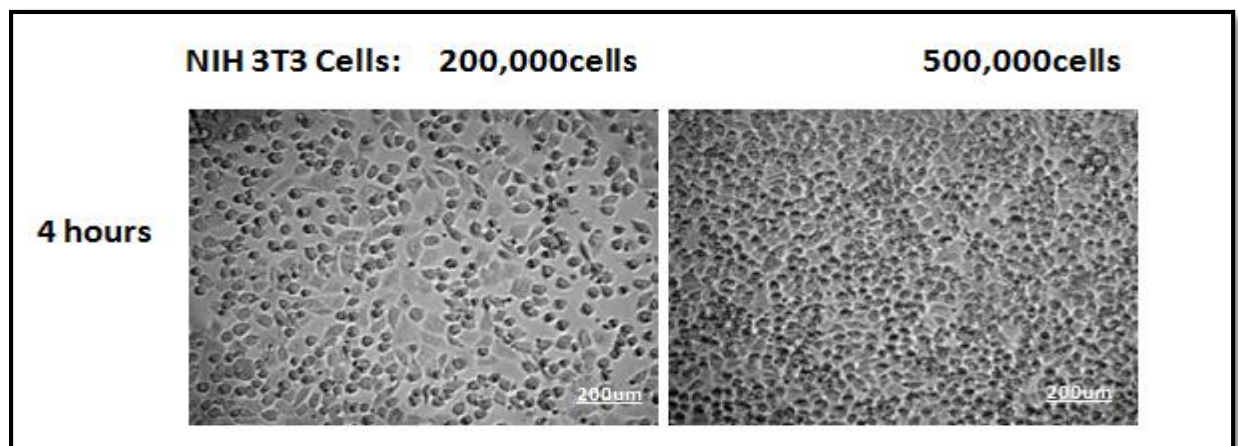
*Figure 11: Outgrowth assay results of NIH-3T3 fibroblast cells. Images are taken at time points 0 hours, 12 hours, and 24 hours. As seen from the photo, NIH-3T3 cells do migrate inward on polystyrene substrate. The vertical white lines mark cell movement from the edge of the scratch towards the middle. The horizontal dashed lines shows how the distance in the middle gets smaller over time as the cells migrate into the middle. This concluded that NIH 3T3 cells will migrate on polystyrene.*

#### 5.1.2 Cell Viability

##### 5.1.2.1 Initial Cell Seeding Concentration

The goal of this experiment was to determine the ideal seeding density of fibroblasts for the assay. NIH 3T3 cells at a concentration of 100,000 cells/200  $\mu$ L of medium were used in the mock assay, and 200  $\mu$ L was placed in the well. This cell concentration was not high enough to

form a confluent layer of cells after 24 hours. The team would like to reduce the cell seeding time to 4 hours and increase the confluence to at least 80% or if possible, 100%. Figure 12 details the experiment used to determine the initial seeding concentration of cells, where the same area was used and the amount of medium used for the mock assay was maintained. In the left picture of Figure 12, 200,000 NIH 3T3 are seeded and to the right 500,000 NIH 3T3 cells are seeded. The cells were incubated at 37 degrees Celsius and 5% CO<sub>2</sub> for four hours and the medium recipe for NIH 3T3 fibroblasts can be found in Appendix F.



*Figure 12: Two seeding concentrations of NIH 3T3 cells after four hours used to compare and determine the ideal seeding concentration. The ideal concentration to seed NIH 3T3 fibroblast cells is at 500,000 cells. This will allow 100% confluence of cells which is desirable for this experiment.*

Analysis of the pictures in Figure 12 showed that for NIH 3T3 fibroblasts, the ideal initial seeding concentration to allow for 100% confluence after four hours was 500,000 cells. Note that if the validation experiment was to be completed with any other cell type, the above experiment would also have to be performed a second time to determine initial cell seeding concentration of the new cell type in relation to the parameters of the assay.

#### *5.1.2.2 Sterilization of Components*

Each component of the design that was intended to come into contact with cells was sterilized effectively. Some of the methods utilized to sterilize materials include autoclaving,



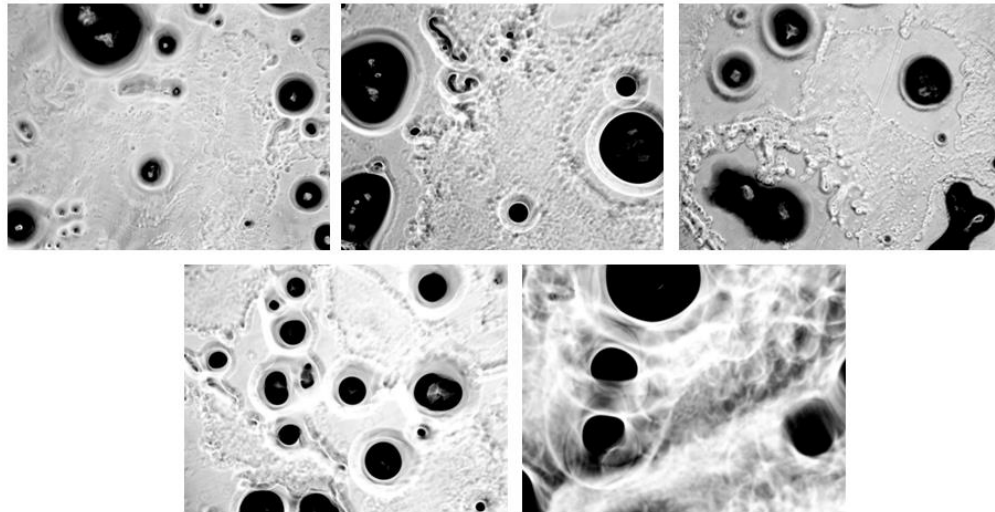
ethylene oxide (ETO), and the use of antibiotics. Autoclaving is a process that utilizes a high heat and moisture environment to kill bacteria. ETO is a chemical process that also kills bacteria, and is utilized in situations which autoclaving cannot perform. However, ETO poses a risk of leaving residuals. Both of these sterilization methods can induce changes in the protein chemistry and physical properties of collagen, and potentially affect absorption rate, mechanical integrity, and performance. Sterilization via autoclaving, however, is a safe method for PDMS, and thus the PDMS molds were sterilized in this way. Because autoclaving would denature collagen and ETO may leave residuals, the collagen gels were sterilized using antibiotics. Collagen gels were harvested and placed into six well plates, and an antibiotic solution was then added to each. The plates were then incubated for eight hours and subsequently rinsed and seeded with cells.

## 5.2 FGF-2 Immobilization Technique

### 5.2.1 Gelatin Microspheres

The team originally utilized gelatin microspheres as a controlled release mechanism for delivering FGF-2. The fabrication process used to fabricate the gelatin microspheres can be found in Appendix F. Gelatin microsphere fabrication, though successful, had limitations. One of the main limitations to the fabrication process was collecting the microspheres into the beaker after formation. Next, the olive oil was removed by adding acetone and centrifuging the microspheres down. Microspheres were then re-suspended in acetone. At this point, the acetone and microsphere mixture was centrifuged again and the supernatant solution (acetone) was aspirated off the top. This left microspheres in a pellet form at the bottom of the centrifuge tube that needed to be transferred to a beaker for drying. However, when they were transferred to the beaker, many of the microspheres remained stuck to the bottom or on the sides of the centrifuge tubes. During the fabrication process six centrifuge tubes were filled with oil and gelatin

microspheres. This should have yielded a significant amount of particles; however, due to the limitations above approximately 75% of the microspheres were unable to be transferred for drying. Figure 13 below shows the gelatin microspheres that were formed. Only a small portion of the microspheres were imaged, but this proved the feasibility of their fabrication was insufficient.



*Figure 13: Gelatin microspheres shown with bright field imaging at 10X. Microspheres were fabricated via a water in oil emulsion technique.*

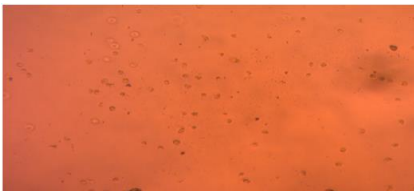

### 5.2.2 Microspheres to Heparin Binding Rationale

As shown from the limitations in section 5.2.1, gelatin microspheres as a controlled release mechanism were deemed inadequate for the design. As seen from the design matrices in Appendix E, heparin binding to FGF-2 recorded the second highest score. Since the cost of heparin was relatively cheap and the protocol for fabricating heparinized FGF-2 sponges realistically feasible within the time constraint, it was chosen as the second design alternative.

## 5.3 Data Collection Technique

### 5.3.1 Inverted Microscope Feasibility

The goal of the inverted microscope feasibility experiment was to determine the ability to image fibroblasts in the collagen gel and collagen-GAG sponge components of  $\mu$ DERM. In Figure 14, cells can be clearly seen in the bright field images of the collagen gel, but cannot be seen in the collagen-GAG sponge. The large black areas seen on the collagen sponge indicate the porous structure of the material. There are many layers to the collagen-GAG sponge, making imaging particularly blurry due to a poor ability to focus the microscope between layers.

<b>300,000 NIH/3T3 cells/100 <math>\mu</math>L plated on materials:</b>	<b>Inverted Microscope (10X)</b>
Collagen gel	
Collagen-GAG sponge secured on top of gel	

*Figure 14: Brightfield images after 24 hours of NIH/3T3 cell incubation on collagen gel and collagen-GAG scaffold on collagen gel. This figure shows that it is not possible to image cells on the collagen/GAG sponge unless additional dye markers are utilized.*

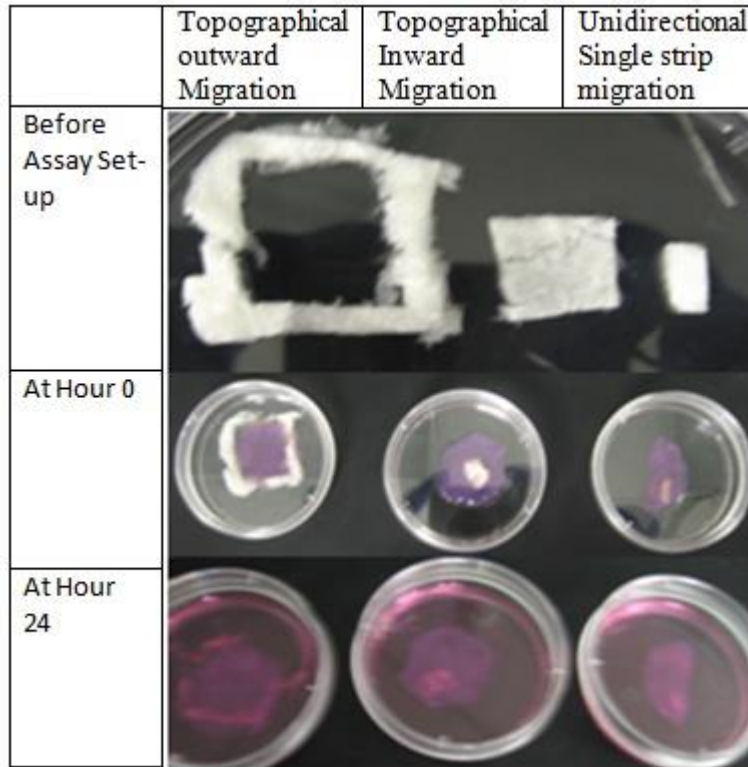
In conclusion, the second imaging idea was used. This imaging idea included the imaging of an un-seeded section of gel between the seeded cell population and the collagen-GAG sponge to measure endothelial cell migration.

A limitations of this experiment was the lack of green fluorescence protein (GFP) marked cells or fluorescently stained cells, which could have aided in the visualization of cells in the

collagen-GAG scaffold. Another limitation was the usage of an inverted microscope for imaging, which images through all the layers of the gel and scaffold instead of imaging from the top down. Since the cells were plated topographically, the potential to see clear images of cells decreased with the increase in translucent layers.

### 5.3.2 Non-Destructive Data Collection

The three non-destructive assays tested were topographical outward, topographical inward, and unidirectional single strip migration assays. Figure 15 displays the experimental design of the topographical outward, inward, and unidirectional single strip migration assays. In Figure 15, the row of images titled ‘before assay set-up’ show the lack of ease of assay set-up in the topographical outward migration. This was because it was challenging to cut the inner square cutout without tearing the scaffold or making uneven edges and corners. It took the least amount of time to cut the unidirectional single strip scaffold. In terms of material conservation, the unidirectional single strip scaffold utilized the smallest area. This decreased costs of running an assay and allowed for more assays to be run at the same time due to the decrease in assay size. At time zero, assay mechanical integrity was good, but by hour 24 the topographical outward migration assay showed signs of poor mechanical structure because one of the sides of the assay fell off the collagen gel.



*Figure 15: Pictures of topographical outward, topographical inward, and unidirectional single strip migration assays at stages of set up, zero, and 24 hours. These experiments were conducted to help determine the mechanical properties of the scaffold when subject to the conditions of the assay.*

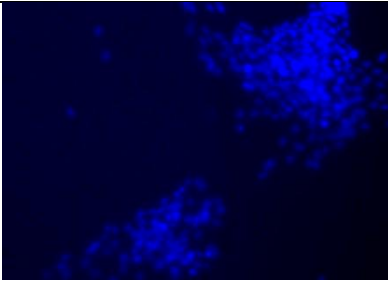
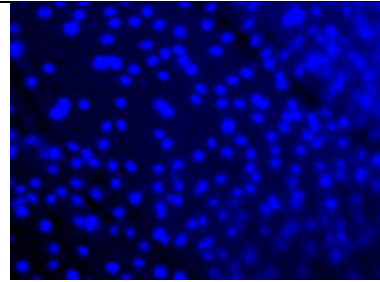
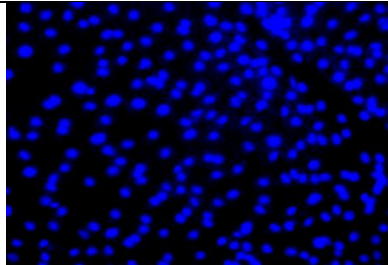
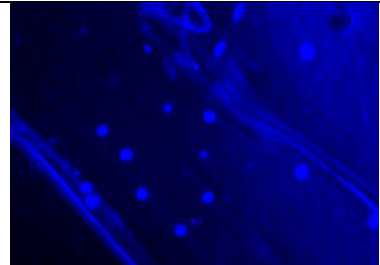
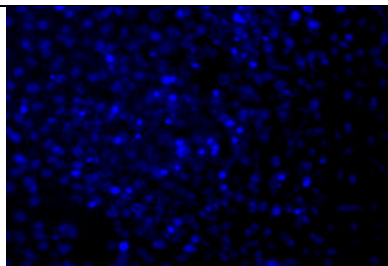
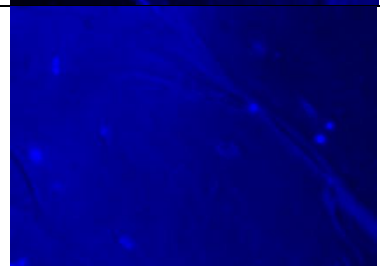
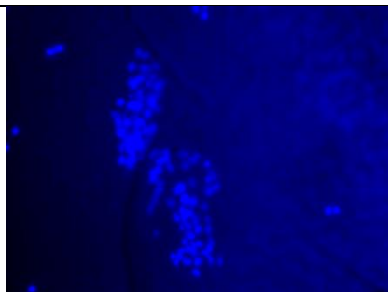
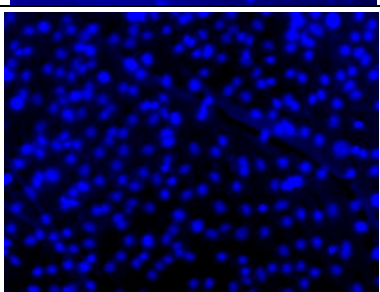
In conclusion, the best non-destructive assay is the unidirectional single strip migration assay as it has good ease of assay set-up, good long term mechanical integrity, and uses the least amount of material increasing the ability for high-throughput assays. The team was able to further modify the unidirectional single strip assay as detailed in section 6.1.5 to further increase the high throughput ability of the final design.

### 5.3.3 Imaging on Collagen Coated PDMS Well

The purpose of this imaging experiment was to gauge the image quality and cell viability in a 10 mm or 5 mm wide and 3 mm deep well environment with a collagen gel coating. The control was the PDMS well without collagen. Once the PDMS molds were fabricated and

sterilized using an autoclave machine, the base of the wells were coated with collagen gel. The collagen was added to the wells using a syringe. Depending on the size of the well, different amounts of collagen were added. The goal was to coat the bottom of the well with a thin layer of evenly spread gel. Once the gelation process is complete, the gels were soaked in antibiotics for 8 hours, then washed three times with Dulbecco's Modified Eagle Medium (DMEM). The cells were then plated at  $5 \times 10^6$  NIH/3T3 cells/mL and incubated for three hours at 37 degrees Celsius and 5% CO<sub>2</sub> to allow for cells to adhere. After three hours of incubation, additional medium was added covering the entire material, and incubated for 24 hours at 37 degrees Celsius and 5% CO<sub>2</sub>. The cells were post-stained with 20 µg of 1 mg/mL Hoechst in 1 mL of medium.

These wells were imaged using fluorescence microscopy and analyzed quantitatively to determine if imaging was possible on wells of this size. Figure 16 shows the images collected from both the sides and middles of the wells.

<b>0.5 x 10<sup>6</sup> NIH/3T3 cells/<math>\mu</math>L plated on materials:</b>	<b>DAPI Imaging of Side of Well</b>	<b>DAPI Imaging of Middle of Well</b>
Well 1- 10 mm width and 3 mm depth, with collagen gel		
Well 2- 10 mm width and 3 mm depth, PDMS		
Well 3- 5 mm width and 3 mm depth, PDMS		
Well 4- 5 mm width and 3 mm depth, with collagen gel		

*Figure 16: DAPI images after 24 hours of NIH/3T3 cell incubation on collagen gel and PDMS. Column two represents images taken on the side of the well at different well dimensions. Similarly, column three represents images taken in the middle of the well at different well dimensions.*

The concern that diffraction would increase background light was minimal in all images. The images showed cell viability in both PDMS and collagen gel coated PDMS, but in different

locations in the well. The PDMS had viable cells congregated at the edges of the wells and significantly fewer cells in the middle. The cells of collagen gel coated PDMS gravitated towards the middle of the wells. In conclusion, cell viability was better on the sides and middle of the collagen gel than on the PDMS, but both the PDMS and collagen showed good image quality.

#### 5.3.4 Final Preliminary Migration Quantification Strategy

The team discussed the migration quantification strategy for the final assay. Initially, it was decided that scratches were to be placed at defined distances on the bottom of the polystyrene dish using a metal blade and ruler. These scratches would be used as initial markers for cell migration when imaging. Details on the design process for quantification of migration and the final quantification design are described in section 6.1.6, the data collection procedure.

### 5.4 Assay Fabrication

#### 5.4.1 Preliminary Design

##### *5.4.1.1 Description*

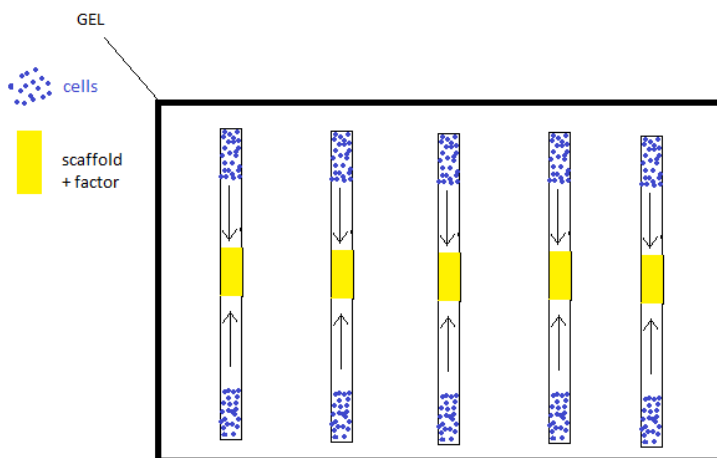
The preliminary design chosen was a PDMS mold with miniature wells coated with collagen gel and plated with cells at either end. The scaffold with implanted FGF-2 gelatin microspheres was placed in the center of the well. This preliminary design concept can be found in Appendix H. The design would allow for high throughput and non-destructive data collection of endothelial cell migration towards the collagen-GAG scaffold imbedded with FGF-2 gelatin microspheres.

##### *5.4.1.2 Computer Aided Design*

To fabricate the preliminary design as explained in section 5.4.1.1, CAD drawings were produced using SolidWorks to create a mold that could be used to make a pathway model out of PDMS. This section outlines the progression of CAD drawings and their limitations which



eventually led to the final design that was rapid prototyped. Figure 17 is a schematic of the concept behind the pathway design assay.



*Figure 17: Computer drawing of fundamental concept behind pathway design assay to quantify endothelial cell migration. Cells were seeded at the edges of each pathway with scaffold containing angiogenic growth factor in the middle of the pathway. Cellular migration could be imaged in the space between cells and scaffold.*

The team determined that this assay design was best suited if it could fit into the well of a six well plate. This would allow for multiple models to be plated at once to optimize the number of data points that could be quantified. Six well plates were measured to have the following dimensions: 17.4 mm depth, top diameter of 35.4 mm, and bottom diameter of 34.8 mm. These dimensions set the parameters for the dimensions selected for the computer aided design models. The first model created in SolidWorks can be seen in Appendix I.

The design was a mold with dimensions 34 mm wide x 34 mm long x 15 mm depth. This mold created a PDMS model with the dimensions of 30 mm wide x 30 mm long x 10 mm depth. This would allow the PDMS model to fit into a standard six well plate. This model consists of five migration pathways that are 1 mm wide x 28 mm long x 1 mm depth. A collagen coating would also be implemented on the surface of the PDMS to allow for endothelial cell attachment.

These pathways are the area in which cells would be seeded and the scaffold would be added.

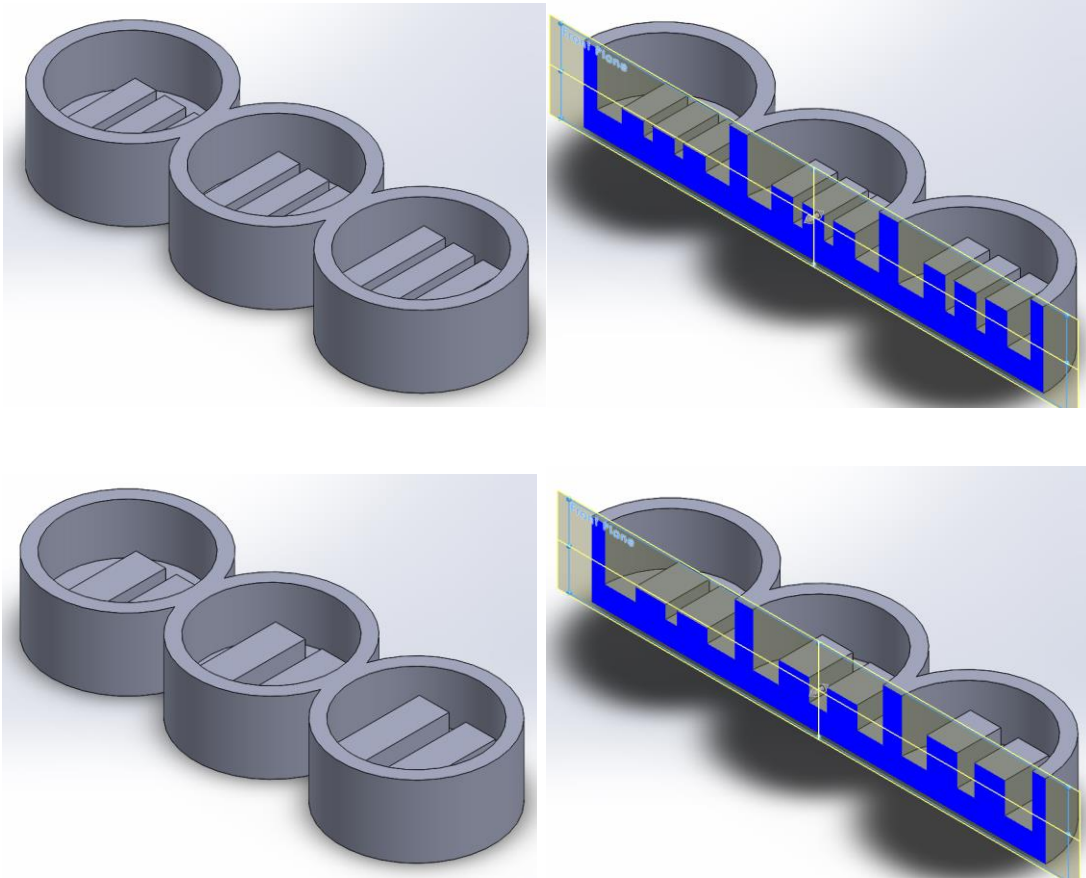
These channels would also hold the medium to be supplied for the cells.

This design was then evaluated by the team and limitations of the design were analyzed. The team realized that this design was not feasible as rapid prototyping or machining (aluminum parts) could not be achieved with 1 mm cuts. This led the team to investigate new mold models that would not violate any of the constraints of machining or rapid prototyping.

The second model that was designed using CAD incorporated a different plan of attack to allow for larger migration pathways that did not violate rapid prototyping or machining constraints. The intent of this model was also to produce molds of PDMS that could be placed into a six well plate. The second model created in SolidWorks can be seen in Appendix J.

The second model created was an improvement compared to the first design. This model had the potential to create six PDMS molds with a diameter of 30 mm to allow placement in a six well plate. This model also allowed the team to analyze the results of each mold to determine the ideal pathway, width, and depth for seeding cells and analyzing migration. The first design produced three molds with a total of six pathways of varying depths and a 9 mm width. The second design produced three molds with a total of nine pathways of varying depths and a 4.5 mm width. These parts were sent to the rapid prototyping machine at WPI where limitations of the design arose. These parts could be fabricated; however, there was a large cost constraint. The two models in Appendix J were estimated to be \$120 which was too expensive for this portion of the project. It was determined that the reason for the large cost estimate was the amount of excess material that was being incorporated into the design. This led the team to reevaluate the design to reduce material and create a third model using CAD.

The third model of the design incorporated similar concepts as the second CAD design; however, it incorporated much less material by cutting out the excess. The third model created in SolidWorks can be seen in Figure 18.



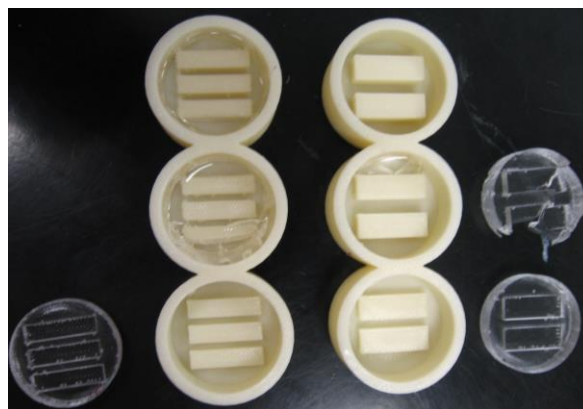
*Figure 18: Third computer aided design models of pathway design. The top two images represent the negative control to make PDMS design of varying depths (3mm, 5mm, and 7 mm) and a pathway width of 5mm. The bottom two images represent the negative control to make PDMS design of varying depths (3mm, 5mm, and 7 mm) and a pathway width of 7mm.*

This design incorporated all the advantages of the second CAD design while also reducing material costs. The design allowed for the team to determine the ideal depth and width that the migration pathway should have and was analyzed for imaging feasibility.

#### 5.4.1.3 Mold Fabrication Technique

The final CAD drawing of the mold for the final design from section 5.4.1.2 was sent to the rapid prototyping lab and the machine shop for fabrication. The team felt that two versions of the mold would be beneficial in that each provided varying migration pathway dimensions. Rapid prototyping was faster and made smaller parts than machining could have produced, but rapid prototyping was expensive and caused rigid pathways from fabrication. These rigid pathways could hinder cellular migration and increase diffraction leading to poor image quality. Machining had a fabrication process that allowed for a smoother finish, minimizing diffraction and not hindering cellular migration as much as rapid prototyping. Machining was also free, but could not be completed in the desired time.

Due to time constraints, the only mold received and validated through testing was the rapid prototype mold. PDMS was made using the protocol in Appendix F. Figure 19 shows images of the PDMS once out of the mold and details some unexpected problems with PDMS casting in rapid prototyped plastic.



*Figure 19: Images of proper PDMS molds at 3 mm depth. The middle row shows 5mm deep molds and the top row shows 7 mm deep molds that were both too deep to remove properly.*

The team was unsure as to the best depth and width of the mold, so the rapid prototyped molds were constructed at 5 mm and 10 mm in width and 3 mm, 5 mm, and 7 mm in depth. The molds with the lowest depth were able to be removed, but only after heating to 60 degrees Celsius and then cooling in a freezer for three minutes to loosen the PDMS from the mold. Molds containing larger depth dimensions were not able to be removed fully from the molds without breaking.

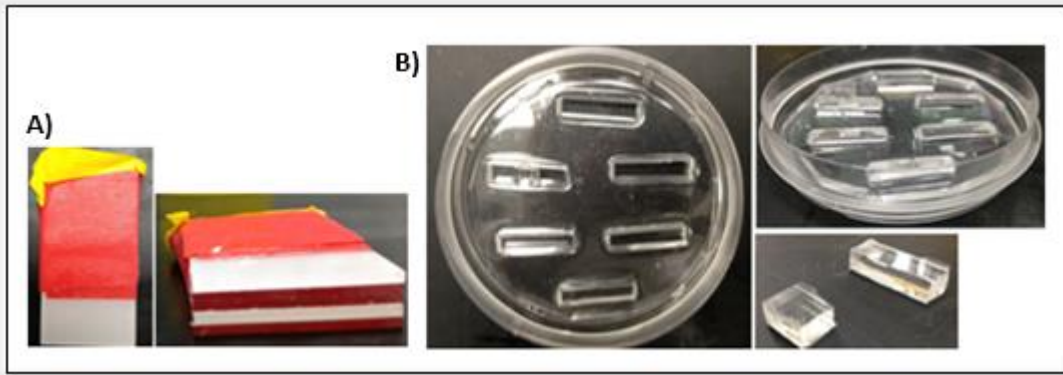
#### 5.4.2 Preliminary Design Changes

##### *5.4.2.1 Converting Assay from Bi-Directional to Uni-Directional*

As discussed in section 4.3.1, the team originally believed that a topographical inward migration assay from two directions would be the most viable design for maintaining the project goals. However, through validation testing, the team realized a unidirectional migration assay was best fit. In the new design, a long narrow well will contain the scaffold and growth factor conjugate on one end and the cells on the other. Cell migration toward the scaffold will be measured in one direction only and any potential concentration gradients of FGF-2 will be negligible. Also, the well will be narrow enough to disallow diagonal cell migration.

##### *5.4.2.2 Changes in PDMS Mold Dimensions and Fabrication*

The previously discussed PDMS mold was too small to allow for PDMS removal without breaking the PDMS. To fix this problem, the team used a Corning® 3295 sterile 60 mm x 15 mm round cell culture dish, with CellBIND® treated polystyrene and made six wells inside this one large round dish.



*Figure 20: A) Five glass microscope slides fastened together to make imprints in PDMS to form wells, B) Post fabrication of slide imprinted PDMS. This shows the final design of well imprints in PDMS along with the PDMS barriers, fabricated in the same fashion as the well imprints.*

Three steps encompassed the PDMS well fabrication process. The first step, shown in Figure 20 part A, was making the glass slides that will hold the form of the wells as the PDMS is poured around them. The glass slides were made of five glass microscope slides from Fisher Scientific (cat#12-544-7) taped together to make a firm, smooth base and sides. Smooth sides were important for the base of the slides so that minimal PDMS gets under the base during fabrication. In addition, smooth slides will form smooth sides of the wells, which will increase image quality when collecting microscope data. The PDMS fabrication procedure can be found in Appendix F. The third step shown in part B of Figure 20 indicates post fabrication steps to prepare the wells where the PDMS was removed from the dish and the inner edges of the wells were scraped to make sure no PDMS appeared at the base of the well. Part B of Figure 20 also shows the PDMS barriers, which were used to separate the cells from the scaffold during the first hours of the assay. The barriers were fabricated in the same fashion as the PDMS wells and followed the same post-fabrication procedure.

### 5.4.3 Feasibility Study of Changed Preliminary Design

#### 5.4.3.1 Mock Assay

After changing the PDMS mold dimensions and fabrication technique, the team verified that the new PDMS wells and barriers would work in the assay by running a mock assay. It was called a mock assay because untreated scaffolds were used and the purpose was for verification of design compatibility with the assay not validation of the final design. Figure 21 details the set-up of the mock assay, specifically part A.

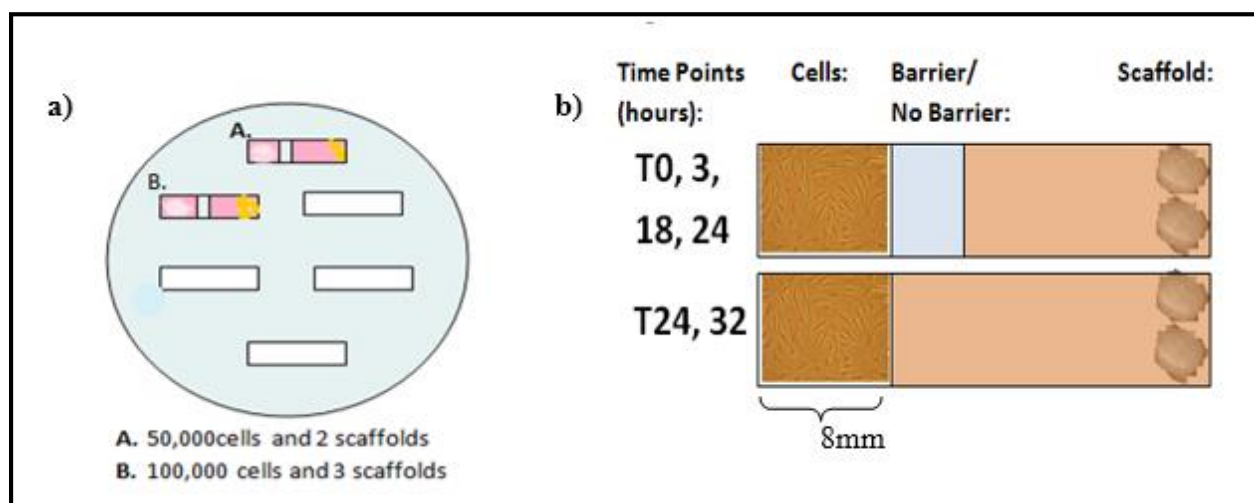


Figure 21: A) Plate layout. Each well was seeded with 50,000 or 100,000 cells at one end. Two or three collagen/GAG sponges were cut and placed at the opposite end of the well. Barriers were set in place at the cell ridge, and the well was flushed with medium. B) Schematic of wells fit to the previously discussed set up in part a.

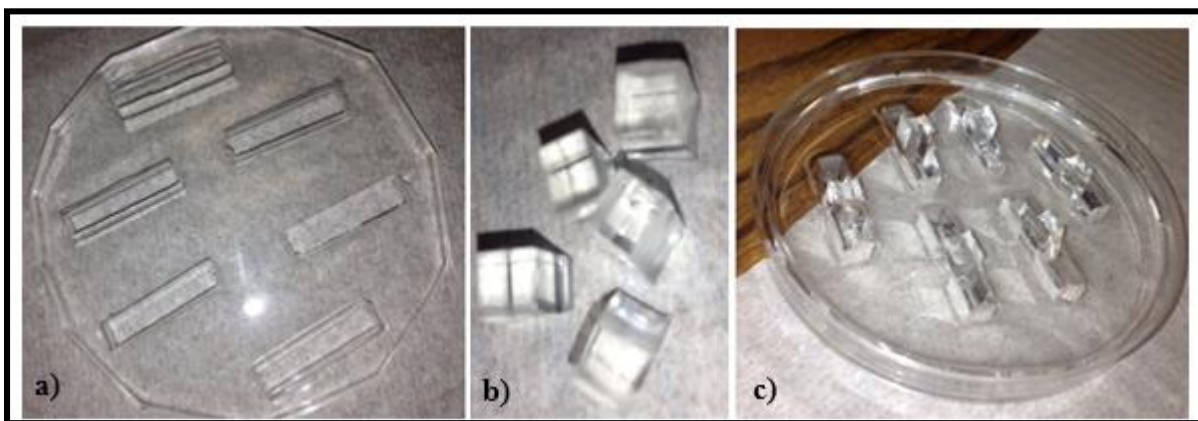
Part B of Figure 21 represents the plate layout showing that in the mock assay only two wells were used. The first well had 500,000 NIH-3T3 cells plated on the left side of the barrier and two scaffolds on the right side. The second well had 100,000 NIH-3T3 cells plated on the left side of the barrier and three scaffolds on the right side. The distance between the left wall and the edge of the barrier was 8 mm for each well to ensure a uniform area for cellular adherence to the polystyrene dish. The scaffolds were tightly pressed to the left wall of the well



to keep them from moving across the well during the assay. The cells were allowed 24 hours to adhere before the barrier was removed.

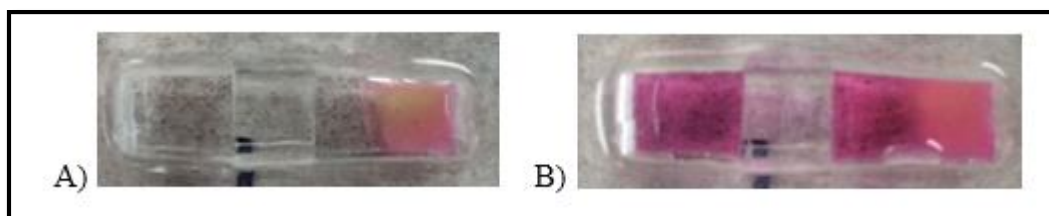
Some concerns regarding the mock assay include PDMS adherence to the polystyrene dish, initial concentration of cells to plate, effectiveness of the barrier in preventing cellular movement, the effect of barrier removal on cellular viability, and adherence of the scaffold to the back wall of the well.

For the mock assay set-up, the outer edges of the PDMS containing the six wells were cut so that the edges of the PDMS would not touch the polystyrene dish, as seen in part A of Figure 22. Cutting the edges decreased resistance on the PDMS from the sides of the polystyrene well, which increased the ability of the PDMS to stick to the polystyrene dish. It was important that the PDMS was completely sealed to the polystyrene well to ensure no medium movement between wells. Additionally, the PDMS was sprayed with ethanol and allowed to air dry to ensure proper sterilization and allow for better adhesion to polystyrene. Part B of Figure 22 shows the PDMS barriers and part C shows how the PDMS barriers were placed in the wells.



*Figure 22: A) Cutting outer edges of PDMS to decrease exterior stresses from the six well plate on the PDMS. This was done to create a vacuum seal of PDMS on the bottom of the polystyrene dish. B) PDMS barrier used to separate the cells from the sponge when the cells are adhering to the polystyrene dish, C) PDMS wells placed in dish and barriers placed in wells.*

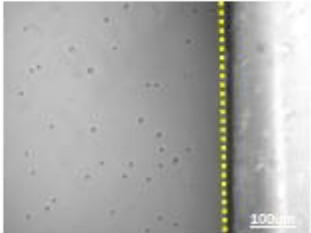
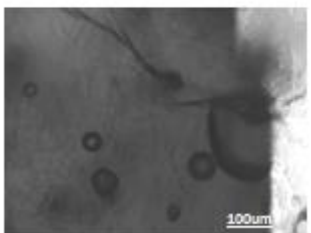
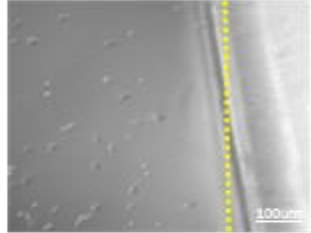
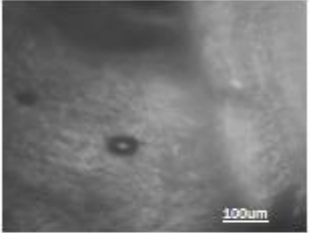
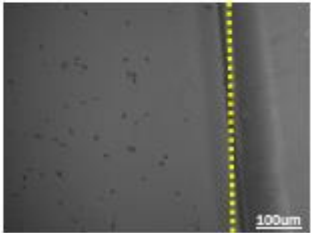
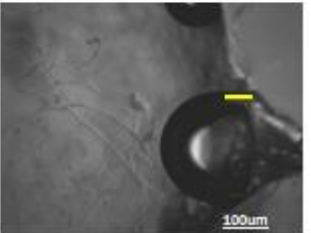
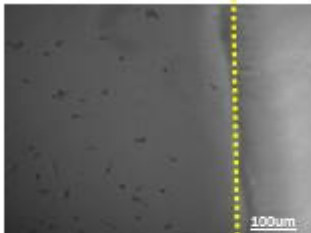
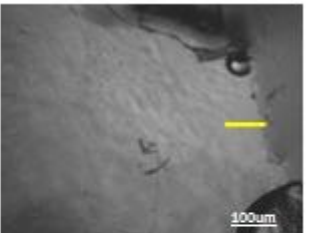
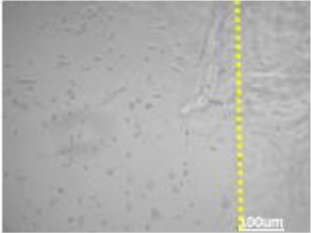
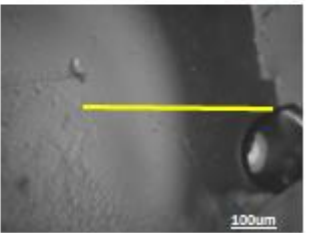




*Figure 23: A) This is an example of adding the scaffold to the wells as the initial assay set up, B) This details where the cells, medium, and sponge are located in the well in relation to the PDMS barrier.*

Once the PDMS wells and barriers were in place, the cells and the scaffold were added, as seen in Figure 23. Using forceps the scaffold was wedged at the end of the well on the right side and 200  $\mu$ L of medium was added. Cells were plated to the left side of the barrier in 200  $\mu$ L of medium. The assay was incubated at 37 degrees Celsius and 5% CO<sub>2</sub> and was only taken out of the incubator to take images at the chosen time points.

Data collected from the mock assay is shown in Figure 24. The first column shows the interface between the cells and PDMS barrier. The images were collected to see if the cells crossed the PDMS barrier. This was important to know because it would affect the accuracy of the experiment if the cells moved beyond the barrier prior to the designated start time. The second column shows the interface between the scaffold and the back right wall of the PDMS well. This data was collected to determine if the scaffold remained attached to the PDMS well wall or if the scaffold detached from the wall and deteriorated across the well. This was important because if the scaffold deteriorated it would come in contact with the cells and negatively impact the accuracy of the experiment. Two images were taken for time point 24, in which the first picture is from pre-barrier-removal and the latter is from post-barrier removal.

Time Points (hours):	NIH 3T3 Fibroblasts (50,000 cells)/PDMS barrier interface:	Scaffold/well wall interface:
T0		
T3		
T18		
T24		
T24		

*Figure 24: Data collected from an outgrowth assay where the NIH 3T3 Fibroblasts are plated at 50,000cells in the designated area between the left PDMS well wall and the PDMS barrier. The scaffold is placed against the right PDMS well wall. The first column is data collected from the cell-PDMS barrier interface and the second column is data collected from the scaffold-PDMS right wall interface at different time points (hours) 0,3,18, 24. The second 24 hour time point shows data after PDMS barrier removal. The dashed yellow line indicates the approximate cell-PDMS barrier interface and the horizontal yellow lines indicate scaffold detachment from the right PDMS wall.*

As seen in column two of Figure 24, the cell-PDMS barrier interface, highlighted by the yellow dashed line, showed no cellular movement across the PDMS barrier. The data showed the PDMS barriers will stop cell movement for up to 24 hours. For the final assay procedure, the team only needed the PDMS barriers to stop cell movement for 4 hours, so this method was adequate. In the second 24 hour time point the PDMS barrier was removed. Again, in the second column of Figure 24, the yellow dashed line indicated the prior cell-PDMS barrier interface and presence of the scaffold. This showed that the scaffold had deteriorated from its initial position at the right well wall to encompass a majority of well. Figure 25 details the extent of scaffold deterioration. The third column in Figure 24 showed that not only is the scaffold deteriorating, it also moved away from the right wall of the PDMS well. The horizontal yellow line indicated the sections of the scaffold that are completely unattached to the PDMS wall and the distance they had moved from the wall.

### **50,000 cells at 24hours with barrier removal (at 4X):**



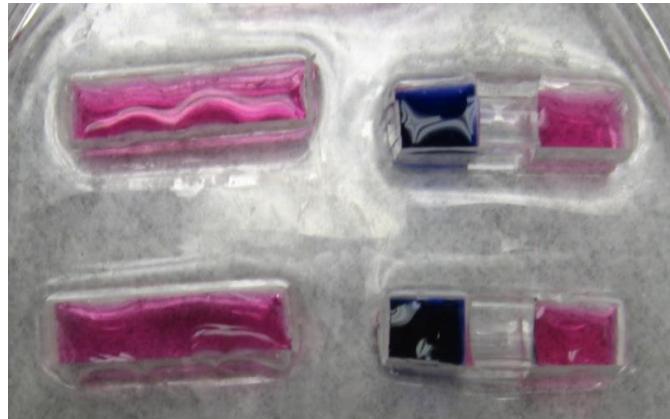
*Figure 25: Overview of experiment at 24 hour time point after PDMS barrier removal. At time point 0 the scaffold is pinned closely to the back right wall of the PDMS well and only takes up one frame. Now at 24 hours it can be seen (above) that the scaffold has deteriorated into 5 frames, which is most of the well. This shows encroachment of the scaffold into the cell region. The long term mechanical stability of the scaffold is lacking. The scaffold must be secured to the right hand wall of the well for experimental success in order for the cells to be undisturbed by scaffold degradation.*

When looking at Figure 25, it can be seen that the scaffold had moved over four frames and was encroaching upon the cell seeded section of the PDMS well. If the scaffold deteriorated across the well, there would be no concentration gradient which would defeat the purpose of the experiment. It was noted that while the PDMS barrier was effective, the scaffold placement

against the back wall the PDMS well was not. The team proceeded to brainstorm design changes to the assay that would allow for the securing of the scaffold to the PDMS wall.

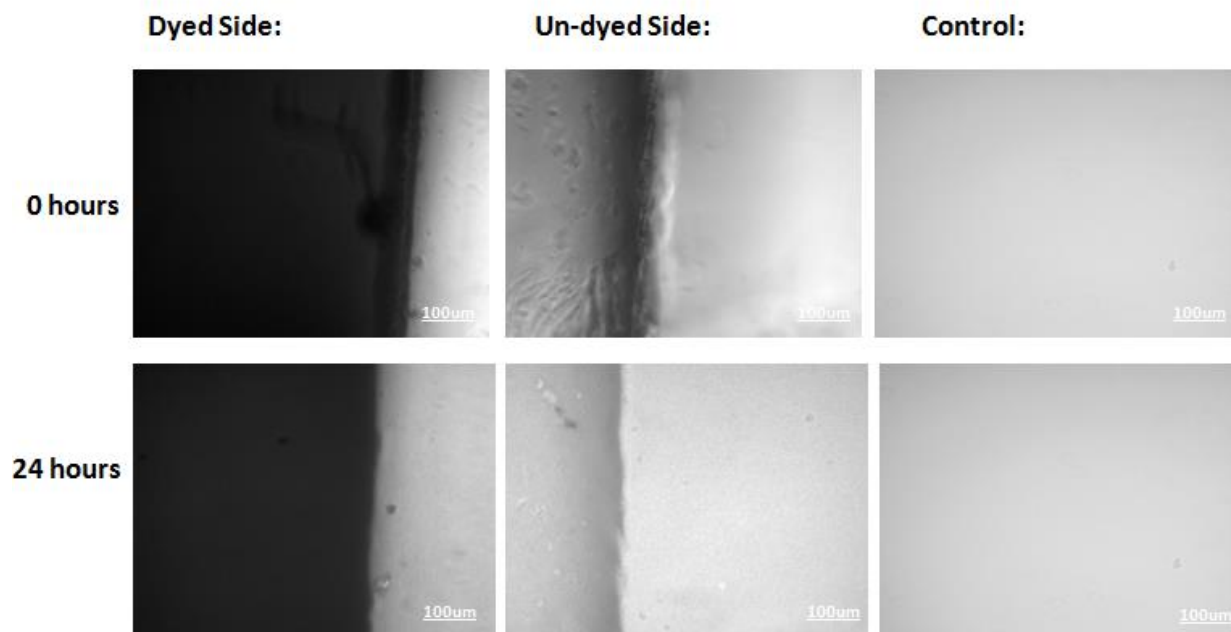
#### *5.4.3.2 Verification of PDMS Barrier Leakage*

The mock assay determined that the cells would not cross the PDMS barrier, but it did not determine if the fluid would cross the PDMS barrier.



*Figure 26: Dye test, imaged after 24 hours, to determine microfluidic movement between the left and right sides. The wells on the left are controls, containing medium only. The wells located on the right are variable wells filled with medium, the PDMS barrier, and Trypan blue stain to determine whether or not leakage was present across the PDMS barrier.*

In Figure 26, the two wells on the left are control wells containing only medium. The wells on the right contain Trypan blue dye on one side and medium on the other side of the barrier. At 0 and 24 hours the wells were imaged, and the images are presented in Figure 27.



*Figure 27: Dye test results indicating no presence of microfluidic movement across the PDMS barrier as data from the controls mimic data of the variable wells. The control is a well with medium and no dye. The control was used to show a comparative control so conclusions could be drawn on other conditions regarding medium leakage across the PDMS barrier of the well.*

Figure 27 showed that the side that did not contain Trypan blue matched with the control at both time points. This indicated that even after 24 hours there was no dye movement into the other side of the well, therefore there was no leakage between the left and right sides of the barrier.

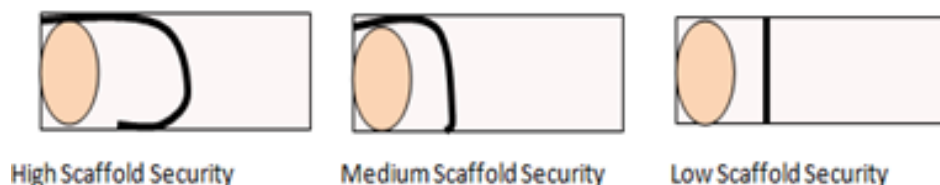
#### *5.4.3.3 PDMS Adhesion to Polystyrene*

The team researched sterile ways to adhere PDMS to polystyrene. One method was to use A-100 medical silicone adhesive or vacuum grease, which can be found in a local hardware store. Vacuum grease dries clear and when properly sterilized produced no cytotoxic effects. The team chose vacuum grease as an adherent method because it was already available in the laboratory. The grease was placed into a syringe and the syringe was autoclaved. Then, a small amount of grease was syringed on the bottom of the PDMS. A thin razor edge was used to spread

the grease over the entire bottom of the well. Any excess grease that fell into the wells was removed using the corner of the razor. Next, the PDMS was placed on the polystyrene and light pressure was applied. The entire plate was placed under ultra violet (UV) light for 20 minutes to complete the sterilization process.

#### 5.4.3.4 Containment of Scaffold

To contain the scaffold against the back right wall of the well, three mechanisms were devised: a high, medium, and low scaffold security gate made of medical grade stainless steel wire mesh. The mesh was small enough to contain the scaffold, but large enough to allow for fluidic movement across the well so the release of growth factors by the scaffold was unaffected. Figure 28 is a schematic of the three different types of scaffold security.



*Figure 28: Scaffold securing mechanisms at high security, medium, and low. A stainless steel, mesh barrier was placed to secure the scaffold to the wall; ensuring no movement of the scaffold throughout the assay.*

The purpose of looking at three different mechanisms was to determine what level of scaffold confinement was needed. The high security barrier gate wrapped entirely around the area where the scaffold was located and required the most time and resources to produce. The medium security gate wrapped around the top of the scaffold, but the bottom part just touched the bottom of the well. Both the high and medium gates were secured by the top part of the gate attaching to the back wall of the PDMS well. The low scaffold security gate touched the bottom of the well, but not the top of the well and anchored into the sides of the PDMS well.

Gates were fabricated from a stainless steel mesh sheet. The length of the gates varied and were not considered a factor in the migration assay as long as they were big enough to contain the scaffold and to be secured in the PDMS well. All the gates were placed in the wells first, leaving 3 mm of space between the gate and back of the well wall where the scaffold was then added. Once the scaffold was placed in the well, the gates that connected to the back wall were bent over the scaffold using forceps and connected to the PDMS. Medium (200 uL) was added to each side of the barrier to mimic a typical assay set-up and the barrier was then removed after several hours. Images of the wells were taken at zero hours after barrier removal and 24 hours after barrier removal. The data is presented Appendix K.

The data showed that all three versions of the gate were successful in containing the scaffold. For both zero and 24 time points the scaffold was contained on the left side by the gate. At no point did the scaffold cross the gate into the other side of the well. For the final assay, the low security gate was used because it utilized the least amount of materials.

#### 5.4.4 Final Design

##### *5.4.4.1 Final Fabrication of PDMS*

A holder for the glass slides was created and sent to be rapid prototyped. The concept is detailed in Figure 29.

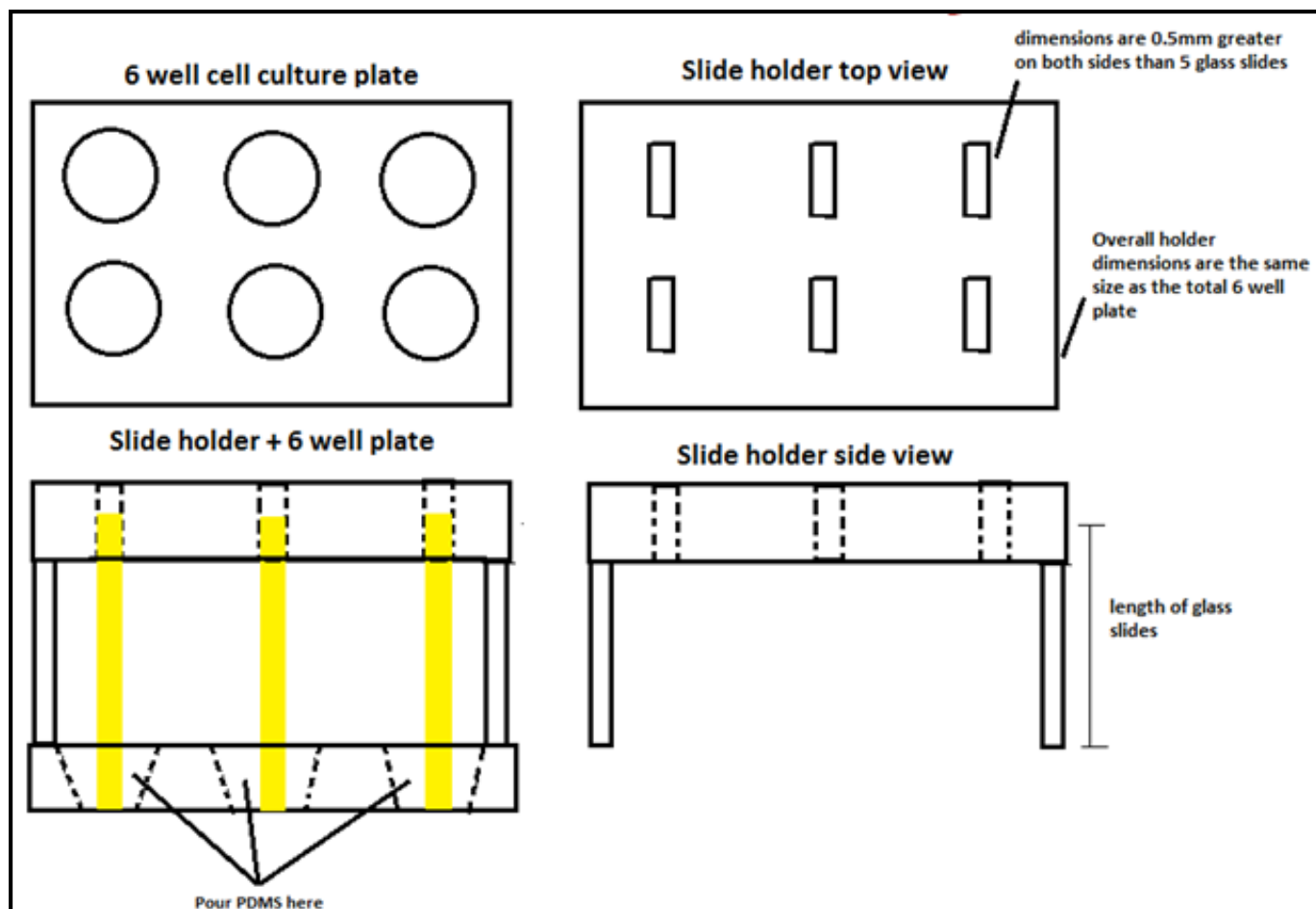


Figure 29: Schematic of rapid prototyped holder for glass slides. The top piece to the rapid prototype is 2.0 cm thick, and the legs are 4.5 cm. The distance between each of the slots horizontally in the rapid prototype piece is 3.5 cm and vertically 1.4 cm. The slot allows a glass slide of 0.5 cm in width to pass through.

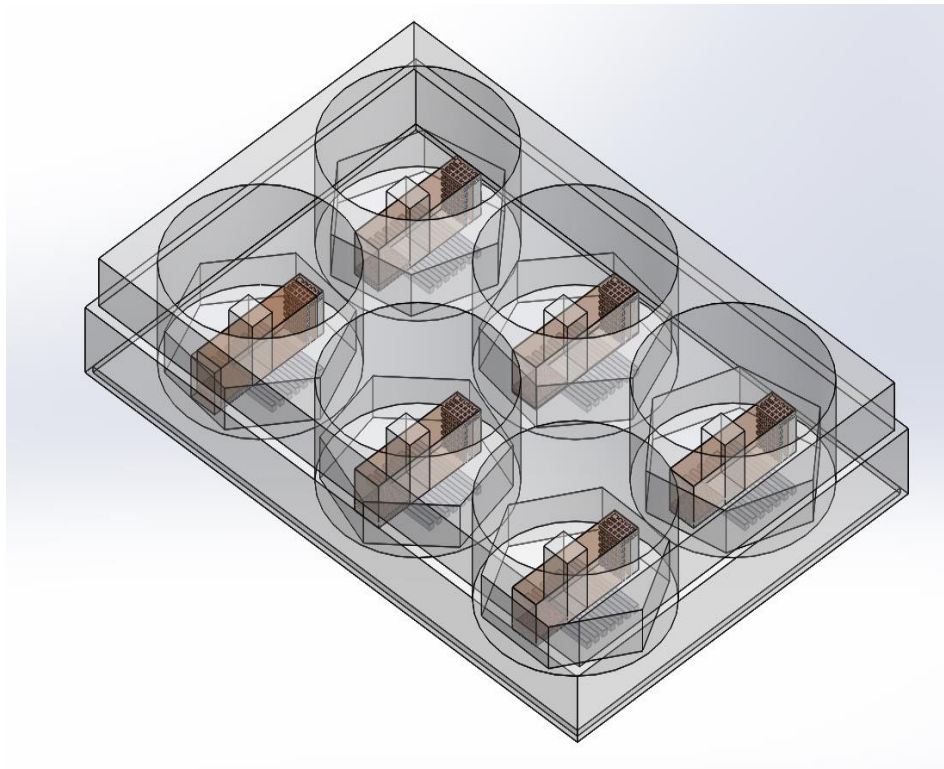
The holder was placed on top of an open six well plate to allow for glass slide placement in individual wells. The glass slides were placed through the slots on the holder and rested on the bottom of the plate. Next, PDMS was poured under the holder and into each well of the plate.

#### 5.4.4.2 Final CAD Design and Description

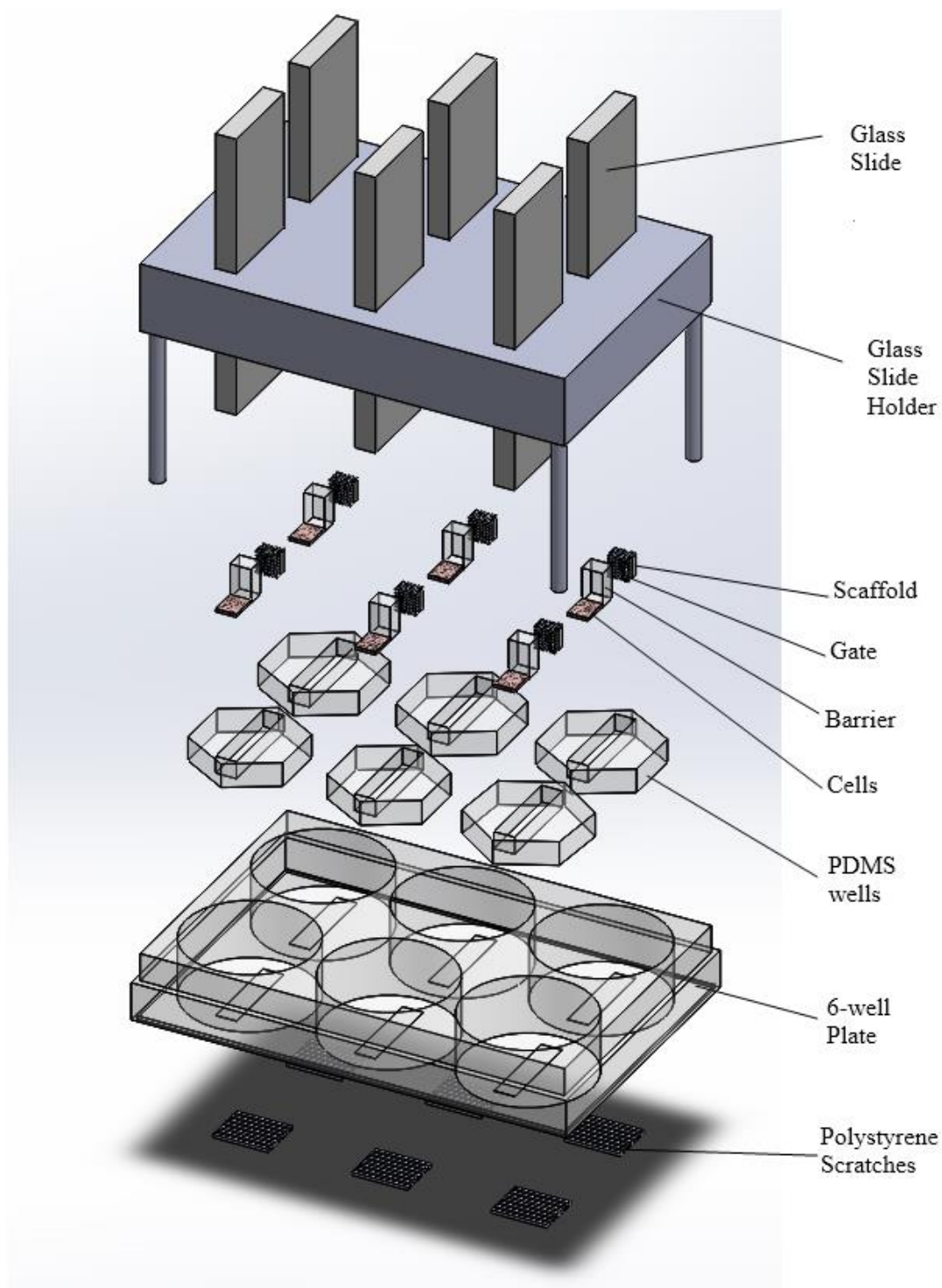
The final design included individual PDMS wells with cells on one end and scaffold on the other end. There was a gate confining the scaffold to keep it in place. In addition, a removable PDMS barrier was used to keep the cells in place and prevent medium leakage prior



to the start of the assay. There were scratches on the bottom of the polystyrene for migration quantification. The final design concept is shown below in Figure 30. Figure 31 is a blown up and labeled schematic of all the parts involved in making the final design seen in Figure 30.



*Figure 30: CAD of final design. This details the placement of the cells in relation to the PDMS barrier, mesh stainless steel gate, and scaffold. Each well is 3.48 cm in diameter and the well channels are 0.5 cm in width and 2.5 cm in length.*



*Figure 31: Schematic of parts comprising the final design. All of the components including the glass slide holder, scaffold, mesh stainless steel gate, PDMS barrier, wells, and polystyrene scratches.*

## 6.0 Validation

### 6.1 Validation Procedure

The final revised design was determined in chapter five. The team took the final revised design and applied it to the experiment set-up. Thus validating the revised final design in the context of the experiment, as seen in the below sections of the experimental procedure.

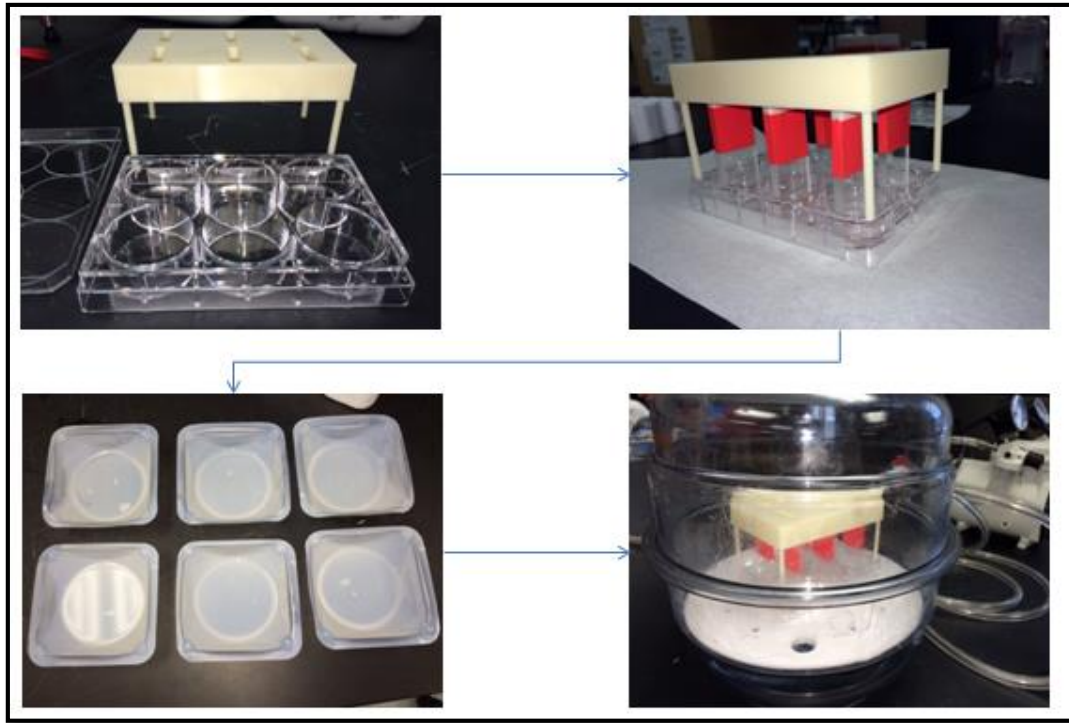
#### 6.1.1 Assay Procedure

The first step was to get the rapid prototyped glass slide holder, as seen in Figure 32.



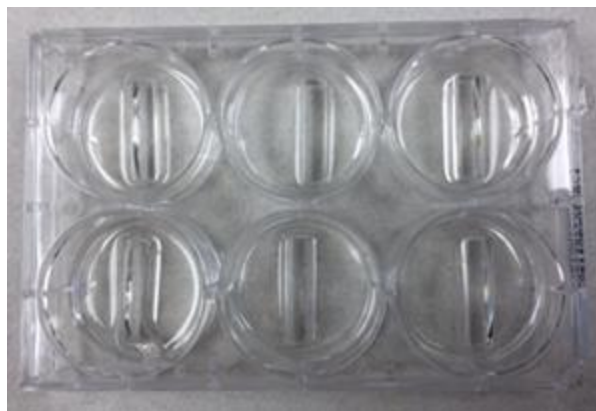
*Figure 32: Picture of the rapid prototyped glass slide holder.*

The rapid prototyped part was placed on top of the six well plate from CELLTREAT (product #229106) and the glass slides were placed through the slots on the glass slide holder. The PDMS was made and poured into the wells on the plate. Figure 33 shows the fabrication of PDMS wells.



*Figure 33: Schematic of final PDMS well fabrication procedure. The glass sides and slide holder were inserted into the six well plate. PDMS was then added to each of the six wells and it was placed in the vacuum chamber for one hour to remove all air bubbles. The arrows indicate the order of the fabrication process.*

After PDMS well fabrication and five minutes in the freezer, the rapid prototyped slide holder was removed and the glass slides were removed from the PDMS. This allowed for easier removal of PDMS from the wells of the plate. First, the glass slides were removed by gently moving them back and forth. The PDMS then detached from the glass and the slide could be pulled out of the well. The PDMS was then removed using a metal spoon and forceps. The edges of the PDMS were then sterilized and vacuum grease was added. The PDMS molds were then and placed in a new six well plate. Figure 34 details the post-fabrication process described above.

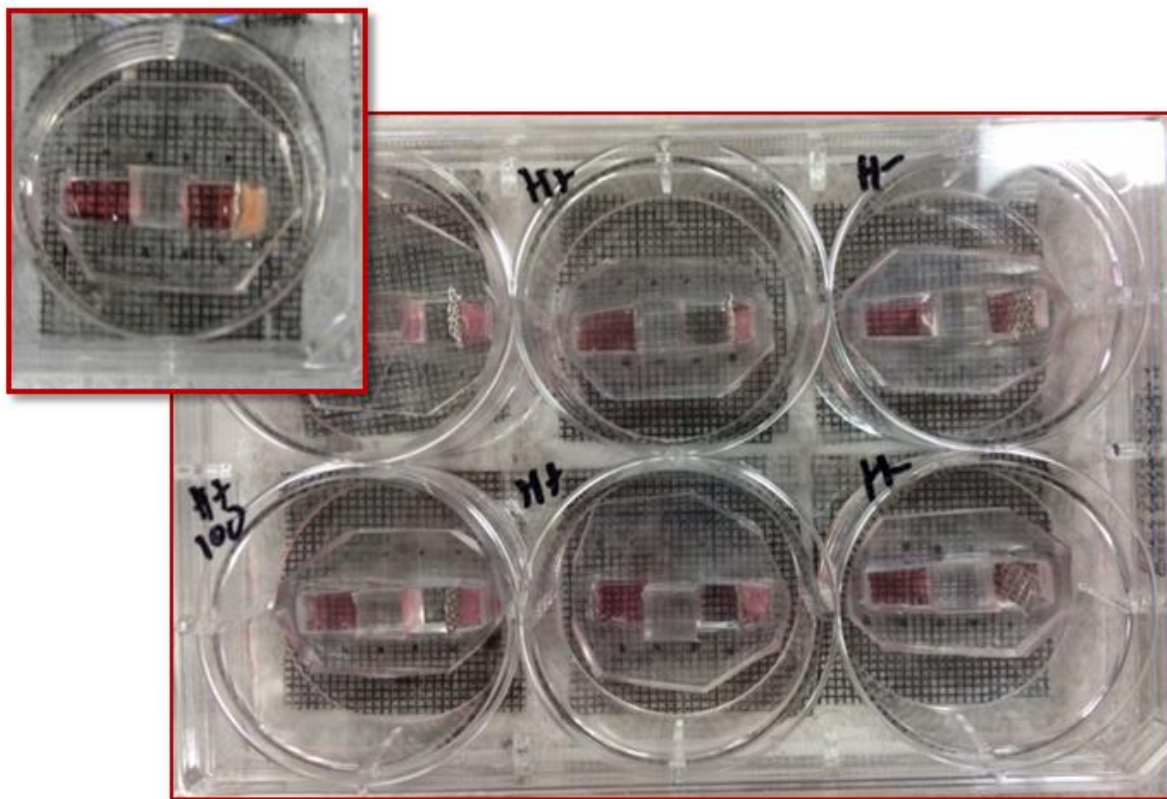


*Figure 34: Schematic of the post-fabrication process for PDMS wells.*

Once the PDMS wells were secured in a new six well plate, the plate was flipped over and scratches were made on the bottom of each well. These scratches would help the team line up the printed grid with the appropriate areas on the well.

The six well plate was then placed upward and the barriers were added 8 mm from the left end of the top of the well wall, and gates were added 3 mm from the right end of the bottom of the well wall. Section 5.4.3.1 details barrier and gate fabrication and techniques for placement in the assay. The lid to the six well plate was then left open under the hood, as the plate was placed under UV light for 20 minutes to sterilize the assay surface.





*Figure 35: Addition of cells, barriers, gates, and scaffold to the assay. One millimeter grid paper was fastened to the bottom of the six well plate to create a grid system to aid in image consistency.*

In a sterile environment, the cells were then added at a concentration of 500,000 NIH 3T3 cells/200  $\mu$ L medium with 200  $\mu$ L being added to the left side of the well. One scaffold was added to each well between the gate and the right wall.

The plates were labeled with permanent marker to indicate different concentrations of heparin and FGF-2 added to the scaffold, as seen in Figure 35. Section 6.1.5 explains the experimental set-up in detail including the type of experimental conditions and how many data collection repetitions of each were performed. After four hours of cell incubation in a 37 degree Celsius and 5% CO<sub>2</sub> incubator, the barriers were removed.

## 6.1.2 Scaffold Preparation

### 6.1.2.1 Heparin Immobilization of Collagen-GAG Sponges

Simultaneously while prepping the assay, the modified collagen-GAG sponges were fabricated. Collagen-GAG sponges were obtained as scrap material and cut into appropriate size using a 9 mm biopsy punch. In order to perform calculations as to how much heparin and FGF-2 were needed for each biopsy, the sponges were weighed and an average weight was determined. The average weight of the 9 mm scaffolds was 1.59 mg and this value was used for all calculations. Table 27 shows the weights of each of the collagen-GAG sponges that were cut using the 9 mm punch biopsy.

*Table 27: This table shows the data for the weights of each of the collagen-GAG sponges that were cut using a 9 mm biopsy punch.*

<b>Weight (mg) 9mm Biopsy Punch</b>	
<b>1</b>	1.6
<b>2</b>	1.3
<b>3</b>	1.3
<b>4</b>	1.5
<b>5</b>	1.8
<b>6</b>	1.4
<b>7</b>	1.5
<b>8</b>	1.9
<b>9</b>	1.9
<b>10</b>	1.5
<b>11</b>	1.8
<b>12</b>	1.6
<b>AVG</b>	<b>1.6</b>

In order to run the experiment six different conditions were given to the scaffolds. These conditions included collagen-GAG sponges that were:

- 1) Non-heparinized with no FGF-2
- 2) Heparinized with FGF-2 (100 ng/mL)
- 3) Heparinized with FGF-2 (200 ng/mL)
- 4) Heparinized with FGF-2 (300 ng/mL)
- 5) Non-heparinized with FGF-2 (300 ng/mL)
- 6) Heparinized with no FGF-2

As seen in the conditions above, four included sponges that needed to be heparinized. Two experiments were to be run per condition using two scaffolds per experiment indicating that sixteen heparinized sponges were needed to be made.

Sixteen collagen-GAG sponge biopsies were incubated in 2-ethanesulfonic acid (MES) buffer solution for thirty minutes in a standard cell culture dish. The MES buffer solution had a concentration of 0.5 M and a pH of 8.0. While incubating each scaffold, a heparin solution was prepared. This solution consisted of heparin sodium salt, EDC, and N-hydroxysulfocinnimide (NHS) at specific molar ratios. The EDC and NHS were used to activate the carboxylic acid groups on the heparin chain to allow for binding to the scaffolds. The protocol for this procedure, as seen in Appendix L, was adapted from a protocol used by Wissink et al.

The following calculations were performed to determine the amount of MES buffer, heparin salt, EDC, and NHS to add to the solution. Since there were eight sponges to be heparinized, the total weight of collagen that was incubating in MES buffer was 25.4 mg (16 sponges).



Next, the amount of MES buffer needed to be calculated. As seen in the protocol, 188.3 mL of MES buffer solution is used to heparinize 1 g of collagen. Using this ratio, the amount of MES buffer needed to prepare the solution was calculated using the equation  $188.3 \text{ mL MES buffer} \times 0.0254 \text{ g collagen}$  which yielded a 4.78 mL volume of MES buffer solution.

Heparin sodium salt was added to the solution first. It can be seen in the protocol that heparin sodium salt is added at 2% w/v to the MES buffer. The following calculation determined the amount of heparin added:  $4.78 \text{ mL} \times 0.02 = 0.0957 \text{ g} = 95.7 \text{ mg}$  heparin sodium salt. From this the amounts of EDC and NHS were calculated using molar ratios as shown in the protocol. The molecular weights for heparin, EDC and NHS used were as follows: Heparin sodium salt (4,000 g/mol), EDC (155.24 g/mol), and NHS (115.09 g/mol). The molar amount of heparin sodium salt was determined using its molar ratio by dividing its weight by its molecular weight;  $0.0957 \text{ g} / 4,000 \text{ g/mol} = 2.39 \times 10^{-5} \text{ mol}$  heparin. Since the molar ratio of EDC to heparin used to make the solution was 0.5, the amount of EDC was half the amount of the molar amount of heparin indicating that  $1.20 \times 10^{-5} \text{ mol}$  EDC should be used. Multiplying this by its molecular weight yielded a weight of 1.86 mg of EDC to be used for the heparin solution. NHS and EDC were then to be added to the heparin solution at a ratio of 0.6 moles EDC: 1 mole NHS. Using this ratio the amount of moles of NHS was determined to be  $1.99 \times 10^{-5} \text{ mol}$  NHS. Multiplying this by its molecular weight yielded a weight of 2.29 mg of NHS to be used for the heparin solution.

Due to the inability to measure small amounts of EDC and NHS (~1 mg) with the scale provided, the team realized this procedure needed to be slightly scaled up. In order to make the heparin solution the following amounts of solution components were used (slightly larger than the calculations); however, the amount of heparin was kept the same in order to maintain the

correct amount for the sixteen sponges. Table 28 displays the amount of each solution component which was added to the heparin solution.

<b>Table 28: The table below records the actual amount of each solution component added to the heparin solution. These amounts are larger than the calculated due to the inability to measure very small amounts using the scale of access.</b>	
<b>Component</b>	<b>Amount Added</b>
<b>MES Buffer</b>	5 mL
<b>Heparin Sodium Salt</b>	96.4 mg
<b>EDC</b>	7.4 mg
<b>NHS</b>	13.6 mg

This heparin solution was left to sit for ten minutes allowing activation of the heparin to occur. Once the solution was ready, the sixteen collagen sponges were transported from the original MES buffer solution to the heparin activated solution using forceps.

The collagen-GAG sponges were allowed to incubate at room temperature in a 24 well plate (16 wells used) containing 400  $\mu$ L of heparin activated solution per well for approximately two hours to allow for heparin binding. Once incubation was complete, the sponges were rinsed in 5 mL of DPBS (-) and then stored in 5 mL DPBS (-) overnight. The eight extra sponges (not containing heparin) were also stored in DPBS (-) overnight in order to prepare for FGF-2 binding and full assay set up.

#### 6.1.2.2 FGF-2 Binding to Collagen-GAG Sponges

Following heparinizing and washing of the scaffolds, they were prepared for FGF-2 binding. As seen by the conditions listed in the previous section, the following scaffolds needed to undergo FGF-2 binding:

- 2) Heparinized with FGF-2 (100 ng/mL)
- 3) Heparinized with FGF-2 (200 ng/mL)

4) Heparinized with FGF-2 (300 ng/mL)

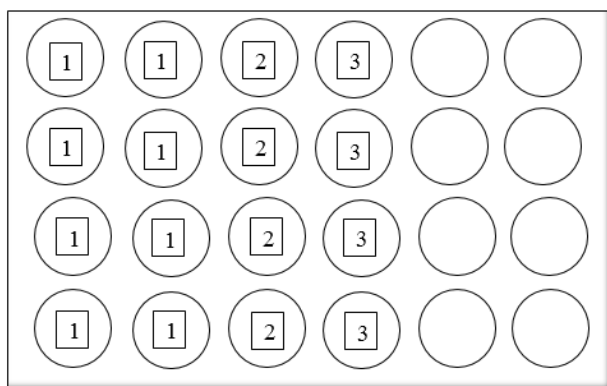
5) Non-heparinized with FGF-2 (300 ng/mL)

This provides six different heparinized sponges at different concentrations of FGF-2 and a non-heparinized FGF-2 sponge. Three separate vials of FGF-2 were used for this experiment. Each vial contained 0.25  $\mu$ L FGF-2 solution at a concentration of 100  $\mu$ g / mL. This was a larger concentration than needed for the experiments and each FGF-2 solution was diluted as follows using phosphate buffered saline (PBS) containing 1 mg/mL bovine serum albumin (BSA). It was determined that all the FGF-2 would be used from each vial and diluted using the appropriate calculated value of PBS solution to provide the correct concentration. First the amount of FGF-2 in the vials (in nanograms) needed to be determined. First, the equivalent amount of FGF-2 in nanograms was determined from its concentration: 100,000 ng / 1,000  $\mu$ L = FGF-2 concentration in vial (100  $\mu$ g / mL). This ratio could then be used to determine the amount in nanograms of FGF-2 in each 25  $\mu$ L vial of solution: 100,000 ng / 1,000  $\mu$ L = x / 25  $\mu$ L (amount of solution in vial) where x = 2,500 ng FGF-2 per vial.

Three concentrations of FGF-2 solution were desired for this experiment; 300 ng FGF-2 / mL PBS, 200 ng FGF-2 / mL PBS, and 100 ng FGF-2 / mL PBS. Since it was determined that there was 2,500 ng in each vial the amount of PBS to add was determined using the following calculations: 2,500 ng FGF-2 / Volume PBS = 300 ng/mL. This provided the amount of PBS needed to prepare a 300 ng/mL concentration of FGF-2 solution. The amount of PBS added to each vial was as follows: 8.33 mL PBS to obtain 300 ng/mL FGF-2 solution, 12.4 mL PBS to obtain 200 ng/mL FGF-2 solution, and 25 mL PBS to obtain 100 ng/mL FGF-2 solution

Each vials contents were micro-pipetted (25  $\mu$ L) and added to the corresponding volume of PBS to obtain the three desired concentrations. Next, 400  $\mu$ L of the FGF-2 solution was added

to a 24-well plate for each of the four conditions (16 wells). Figure 36 is a diagram representing the layout of the FGF-2 binding assay. Wells represented by “1” are designated to 400  $\mu$ L FGF-2 solution at 300 ng/mL, well “2” was designated to 400  $\mu$ L FGF-2 solution at 200 ng/mL, and well “3” was designated to 400  $\mu$ L FGF-2 solution at 100 ng/mL.



*Figure 36: The diagram above shows the set up for the FGF-2 binding assay. The wells labelled “1” were designated for 400  $\mu$ L FGF-2 solution at 300 ng/mL. Wells labelled “2” were designated for 400  $\mu$ L FGF-2 solution at 200 ng/mL and wells labelled “3” were designated for 400  $\mu$ L FGF-2 solution at 100 ng/mL.*

The heparinized sponges were then moved from heparin solution into the FGF-2 solution wells, leaving four sponges behind as heparinized sponges with no FGF-2. The sponges were incubated for 90 minutes at room temperature. All of the sponges for all conditions were then rinsed in PBS, sterilized, and transferred to a new 24-well plate and stored in an antibiotic cocktail to be sterilized. The 24-well plate was then placed in the incubator overnight. The next day, after the sponges incubated in antibiotics for approximately 19 hours, the assay set up was prepared. The sponges were then rinsed three times for 20 minutes in medium to ensure no antibiotics would be present on the sponges. The sterile sponges were then moved into the pathways using sterile forceps.

### 6.1.3 Cell Preparation

NIH 3T3 fibroblast cells (Ambady Lab) were cultured in T75 flasks and passaged once a week. The medium recipe can be found in Appendix F. No special preparation of NIH 3T3 cells was needed for this assay. NIH 3T3 cells were incubated at 37 degrees Celsius in 5% CO<sub>2</sub>.

All the above experimentation has been completed with fibroblasts while waiting for endothelial cells to reach desirable confluence. The assay validation will also be completed with endothelial cells.

Human pulmonary artery endothelial cells (HPAECs) were received from the Pins Lab in a frozen cryovial containing 1x10<sup>6</sup> cells. Endothelial cell growth medium was purchased from LONZA Clonetics (cat # 3024A). The medium included a vial of bovine brain extract (BBE, cat# cc-4092), which was added to the medium. After mixing the two components, medium only lasted for 30 days and was kept in a dark refrigerator or wrapped in foil. The vial of cells was thawed and plated at 375,000 cells/75cm<sup>2</sup> (per T75 flask). HPAEC's were incubated at 37 degrees Celsius in 5% CO<sub>2</sub>.

### 6.1.4 Power Analysis and ANOVA

#### *6.1.4.1 One-way ANOVA Testing for Sample Size*

In order to determine the experimental design, statistical analysis needed to be performed to indicate the number of samples needed for each condition why. Since there were more than two conditions being tested and only one type of data being analyzed, a one way analysis of variance test (ANOVA) for sample size could be performed using the software application SigmaStat 12.5. The following six conditions were tested.

- 1) Non-heparinized with no FGF-2
- 2) Heparinized with FGF-2 (100 ng/mL)

- 3) Heparinized with FGF-2 (200 ng/mL)
- 4) Heparinized with FGF-2 (300 ng/mL)
- 5) Non-heparinized with FGF-2 (300 ng/mL)
- 6) Heparinized with no FGF-2

SigmaStat 12.5 allows prediction of the number of samples needed to obtain significant statistical data for a number of conditions and a confidence level. The following information was needed for the software to perform the calculation: minimum detectable difference between means, expected standard deviation, number of groups, desired power, and an alpha value. The minimum detectable difference and expected standard deviations were values that were estimated through literature. The following values were entered into the program and the predicted number of samples shown.

Minimum Detectable Difference Between Means: **7  $\mu\text{m/hr}$**

Expected Standard Deviation: **5  $\mu\text{m/hr}$**

Number of Groups: **6**

Desired Power: **0.95**

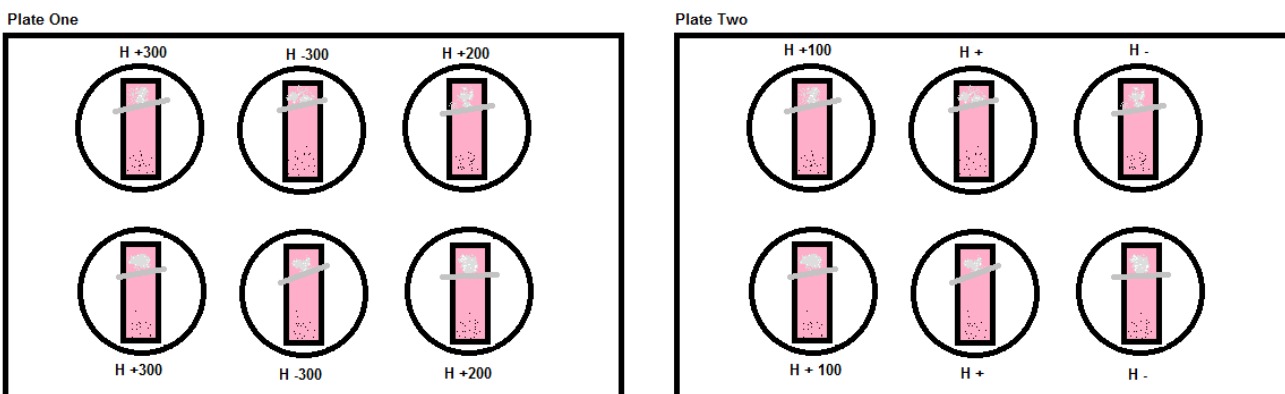
Alpha: **0.05**

The calculated number of samples was determined to be twenty one. Twenty one samples would be ideal; however, due to time and budget constraints as many samples will be generated as possible even if lower than the calculated value.

#### 6.1.5 Experimental Set-Up

As discussed, six conditions (non-heparinized with no FGF-2, heparinized with FGF-2 at 100 ng/mL, heparinized with FGF-2 at 200 ng/mL, heparinized with FGF-2 at 300 ng/mL, non-heparinized with FGF-2 at 300 ng/mL, and heparinized with no FGF-2) were tested on two six

well culture plates. Figure 37 is a diagram of the plate set up of different conditions at different concentrations.



*Figure 37: Schematic of the six well plate set up for the assay design. Six conditions were tested and each condition was tested twice for accuracy. In the schematics of plate one (left) and plate two (right) the rectangular zone inside the polystyrene well is the medium filled column. The media is represented as the pink in each well. The scaffold was seeded on the upper-half of the well, as seen by the white coloring in the pink medium. The barriers were set in place and the cells were seeded on the lower-most half of the well. Plate one represents to trials of the conditions heparinized FGF-2 at positive 300 ng/mL, negative 300 ng/mL, and positive 20 ng/mL. Plate two, respectively, represents the two trials of the conditions heparinized FGF-2 at 200 ng/mL, heparinized with no FGF-2, and non-heparinized FGF-2.*

The team decided to create a “notch” system for quantifying cell migration. Using a small blade, cuts were made on the bottom of the culture plate 0.5 centimeters apart through each well. Each cut, or notch, would help create a distinguished zone of imaging to track cell migration. For example, if cells migrated from notch 0 to notch 0.4 in a specific time period, the zone of imaging in the following time period would begin at notch 0.4. At the end of the experiment, the total notch distance which the cells migrated would be converted to a measurement.

The team shortly discovered the notch system would not accurately quantify cell migration because the notch distances were too large. Cells would not travel through more than one notch, and the exact placement of cells between notches could not be properly measured. Thus, one square millimeter graph paper was photocopied onto clear transparent sheets. The transparent grid sheets were fastened on the bottom of the six well plates in alignment with the

pre-scratched notches under each well. The grid pattern was used as a coordinate system. A thorough description on the usage of the coordinate system may be found in the following section 6.1.6.

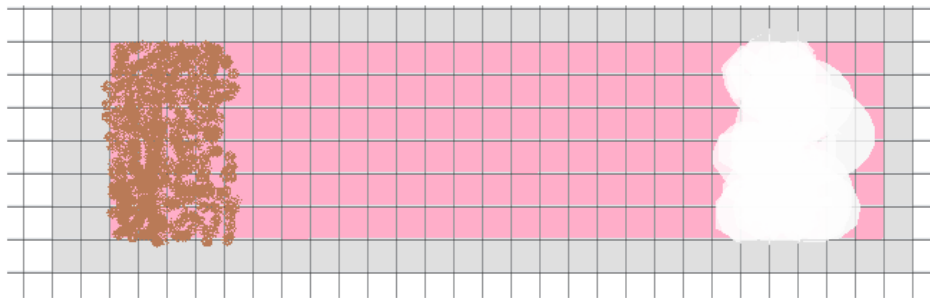
#### 6.1.6 Data Collection Procedure

The team has established a unique and precise procedure to gather data. A study by Rose examined the effect of specific growth factors on equine oral and limb fibroblast cells. Through an *in vitro* bi-directional scratch assay, cell proliferation and migration of fibroblast cells was studied when in conjunction with PDGF and FGF-2 (Rose, 2012). Rose imaged the gap zone of the scratch at time points 0 hours, 5 hours, 10 hours, 24 hours, and 36 hours using Zeiss microscopy and Axiovert 40 CFL (Rose, 2012). The team conducted a uni-directional cell migration assay with a data collection strategy based off the protocol by Rose. However, rather than collecting data every five and twelve hours respectively, the team collected data every four hours. At each time point, two images of each well were taken. This was done to ensure accuracy in the images as well as provide a comparative tool between images. Collecting data every four hours allowed for an ease in scheduling; evenly breaking up the 24 hour day and ensuring every team member had an equal responsibility in the process. Thus, the team collected data beginning at time point zero hours and continued data collection every four hours until time point 100 hours (four days total). The experimental assay was initially performed with NIH 3T3 mouse fibroblast cells to practice collecting data and ensure experimental success. The final assay utilized endothelial cells to support the main objective of creating an *in vitro* assay promoting vascularization and tissue ingrowth of the  $\mu$ DERM tissue engineered skin substitute.

Prior to the start of this protocol, the team created a document to track specific activity throughout data collection. First, the barriers set in place inside each well were removed and



marked to distinguish the initial starting point for cell migration. To begin collecting data, the Zeiss microscope was set to a magnification of 20X. Using the grid pattern on the bottom of the well plate, the specific area of interest – the well- was located. This was done through an analysis of color; the well appeared pink under 20X magnification because of the medium presence while the PDMS appeared a yellow-brown. Figure 38 demonstrates the well under 20X magnification and the location of the cell edge. Once the area of the well was positioned, the coordinates of the barrier removal line were found and recorded at time point zero hours. The coordinates were determined using a simple counting procedure. Grid lines were counted horizontally and vertically to isolate a single square of focus. For example, if the barrier removal line was at coordinates horizontal 6-7 and vertical 3-4, a single square would be isolated between horizontal lines 6 and 7, and vertical lines 3 and 4. The isolated square would mark the starting location of fibroblast cells before migration.

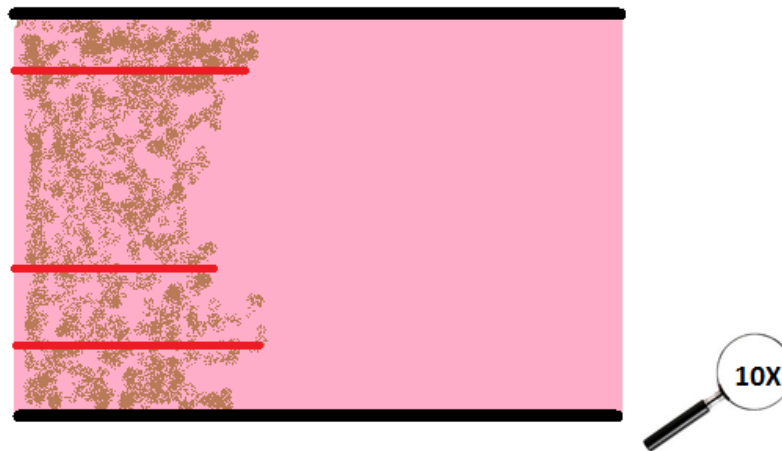


*Figure 38: An illustration of how the well was located under 20X magnification. The medium in the well appeared pink, while the solid PDMS was a grey-brown color. Once the area of the well was located, the zone of imaging was determined. The team examined the cell edge found in the well. This was the rightmost edge of cells, or the starting point of the cells. Once this edge was found, the team chose a particular square along the ridge to begin measuring. The chosen square displayed the cell edge. Then, the team used a simple counting procedure to create a coordinate system. If the starting position had coordinates horizontal 6-7 and vertical 3-4, the square of interest for imaging would be found by counting lines 6 and 7 from left to right, and vertical lines 3 and 4 downward. The zone of imaging is the square formed from the four coordinate lines and was used to locate the imaging zone to ensure each team member was imaging the same “spot” on the cell edge for consistency.*

After isolating the square of interest, the Zeiss microscope was set to a magnification of 10X to display a more magnified image of the cells in that particular zone. Using the software AxioVision, an image was taken to document cell position. An image was taken of each well at each time period and saved in folders following a very specific format. At the start of each time point, the responsible team member would refer to the tracking document to recognize the imaging coordinates found in the prior time period. From this, the team member would use the coordinates and examine the area. If the cells seemed to migrate out of the previous coordinates, the new found coordinates were recorded to determine the new imaging zone to be examined by the next responsible team member.

At the final time period, time point 100 hours, a total of 26 photos were taken per well and 52 photos taken per condition. The team used the photos in conjunction with ImageJ to quantify cell migration. Each image obtained in one well was aligned vertically to show the horizontal migration of the cells after each time period. It was noted that each team member possessed different imaging techniques. Some team members imaged directly between the necessary coordinates, while other team members imaged slightly to the left of the necessary coordinates to show the concentration gradient of the cells as they migrated horizontally. In both situations, the grid coordinate lines were used as reference points. Using ImageJ, a horizontal measurement line was drawn from the leftmost horizontal grid line to the visible resting point of the cells in each photograph. These lines were then converted into measurements. The migration of cells could then be seen by noting the difference in length between two consecutive time points. This measurement will be taken into account with the 100 hour time allocation and a rate will be calculated. Additionally, the grid coordinate system was a second technique used to quantify cell migration. The difference in horizontal grid coordinates was measured by

converting each square into a length. If the fibroblast cells only traveled through one and a half grid lines, for example, the migrated distance is the length of one square, 1 millimeter, multiplied by the distance of 1.5 squares. Figure 39 is a schematic illustrating the quantification strategy described using ImageJ.



*Figure 39: After isolating the imaging zone along the cell edge, as discussed in Figure 42, the team zoomed to 10X magnification. Figure 43 depicts the image of the cell edge in the isolated coordinates in 10X magnification. Two images were taken, and in ImageJ, three lines were drawn from the leftmost grid coordinate to the outermost cell.*

## 7.0 Discussion

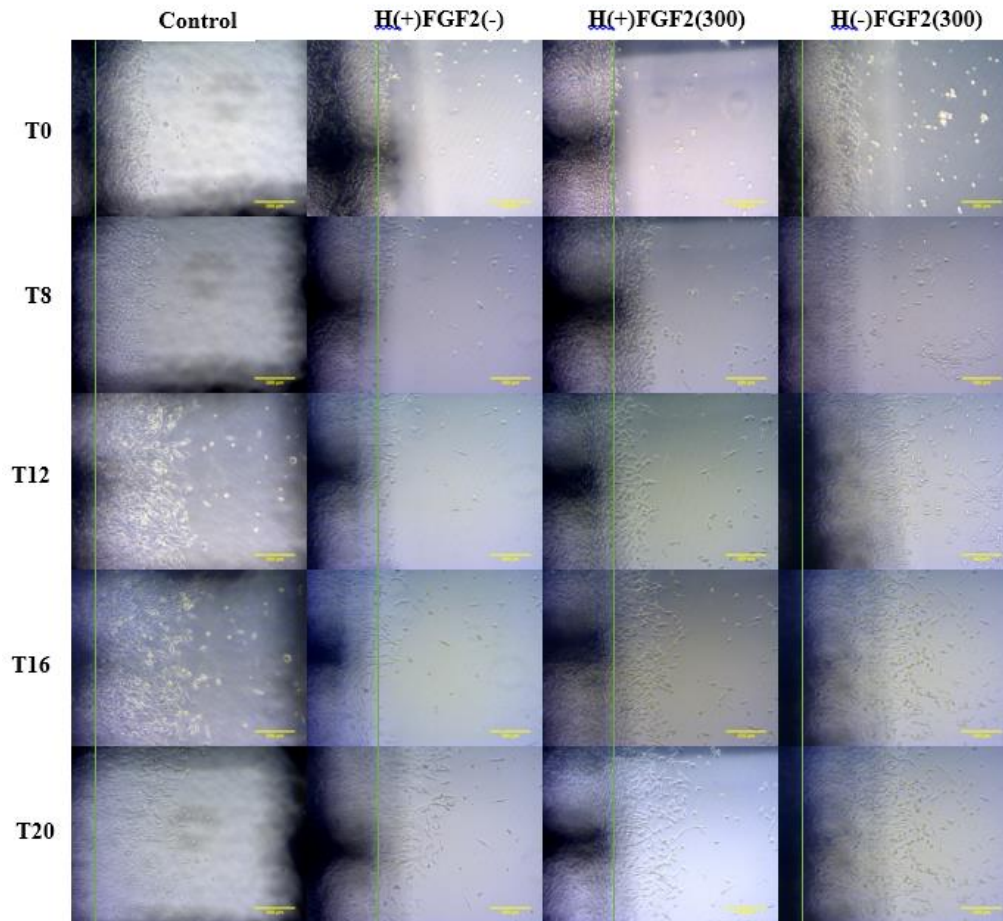
### 7.1 Discussion of Project Changes

Due to time and monetary constraints, fibroblasts instead of endothelial cells are used for the project design validation testing. The use of fibroblasts instead of endothelial cells does not change the variables measured in the validation testing or affect the potential for tissue ingrowth and subsequent angiogenesis. This is because fibroblasts are actually one of the primary cells needed at the commencement of angiogenesis where fibroblasts allow for endothelial cell movement into the matrix by producing collagen, which forms the post- granulation tissue extracellular matrix (Jetten et al., 2014). The migration of fibroblasts into the wound is a rate

limiting step, in that, if fibroblasts are not present, collagen is not produced, endothelial cells will not migrate into the wound, and no angiogenesis will occur. An outgrowth assay to assess fibroblast or endothelial cell migration towards a collagen/GAG sponge with heparin bound FGF-2, will aid in determining if angiogenesis is possible in this application.

## 7.2 Discussion of Obtained Results

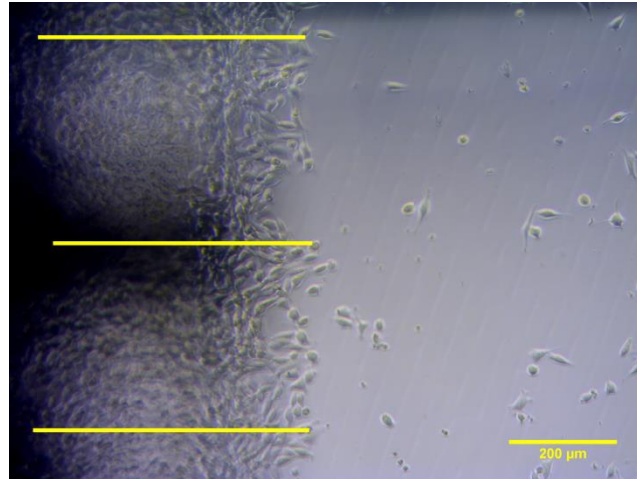
After utilizing the data collection strategy outlined in section 6.1.6, image and data analysis needed to be performed in order to represent and quantify findings from the assay. As discussed previously, images for each condition were taken every four hours for twenty hours after cells were adherent. Image analysis was accomplished utilizing the open source program ImageJ which allows the user to set image scales and perform accurate and reproducible measurements for each image taken. Figure 40 shows the image progression from zero hours to twenty hours for the control, heparin positive FGF-2 negative, heparin positive FGF-2 positive, and heparin negative FGF-2 positive (300 ng/mL). This figure aims to show the outgrowth of NIH/3T3 fibroblasts toward the collagen-GAG sponge under these various conditions. These conditions were shown in particular because it was seen from the analysis that 300 ng/mL of FGF-2 was most prominent in signaling fibroblast outgrowth.



*Figure 40: This figure shows the NIH/3T3 fibroblast outgrowth for four of the six conditions tested. The line in each image represents the relative position of the start location for the cells (T0). The left most images represent the control (heparin negative and FGF-2 negative), the left inner images represent heparin positive FGF-2 negative sponges, the right inner images represent heparin positive FGF-2 300 ng/mL sponges, and the right most images represent heparin negative FGF-2 300 ng/mL sponges.*

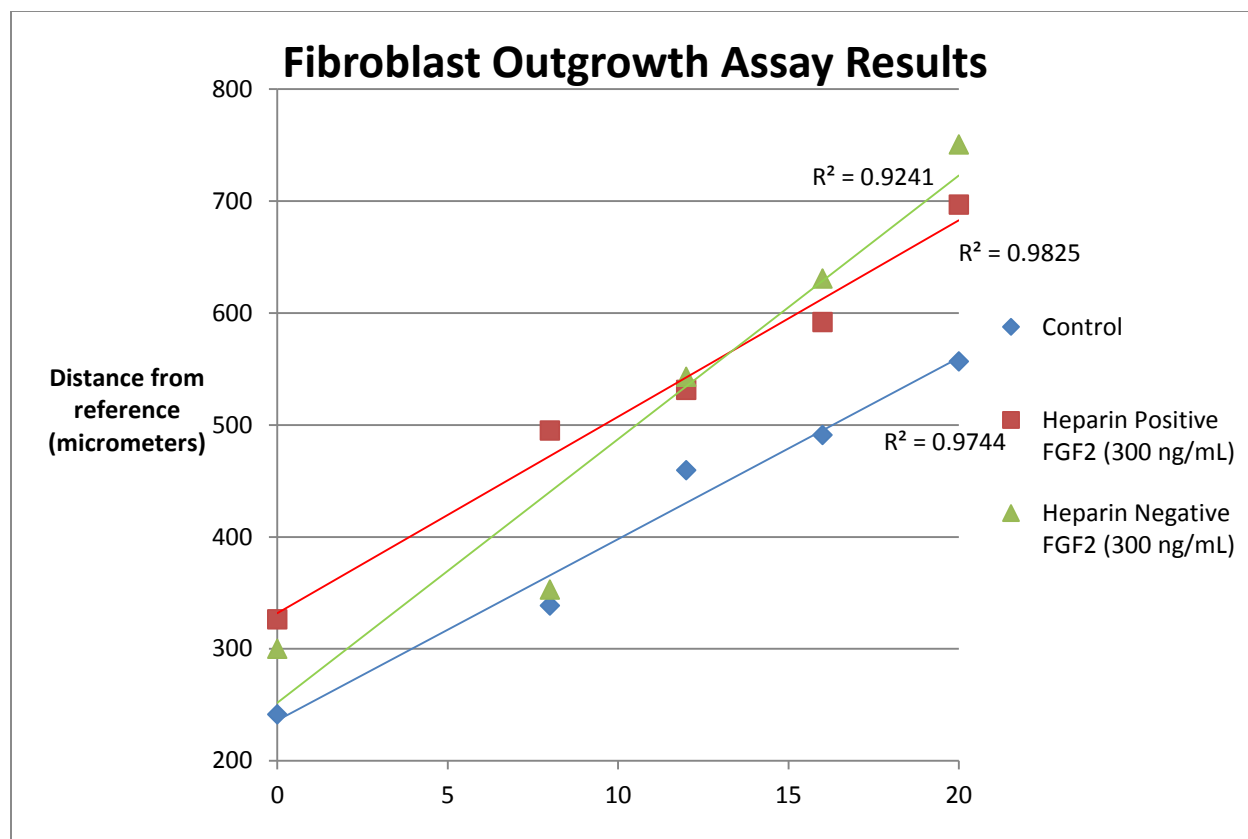
Although only four sets of images are represented above, image analysis was performed on all six conditions for two separate trials. Although not represented in Figure 40, these images can be found in Appendix M. Measurements were made by measuring from the reference point (black line on left of images) to the three furthest points the cells migrated to. These measurements were made using ImageJ and the data were saved to CSV files for each image. Figure 41 shows an example of how the measurements were made.

**T16**



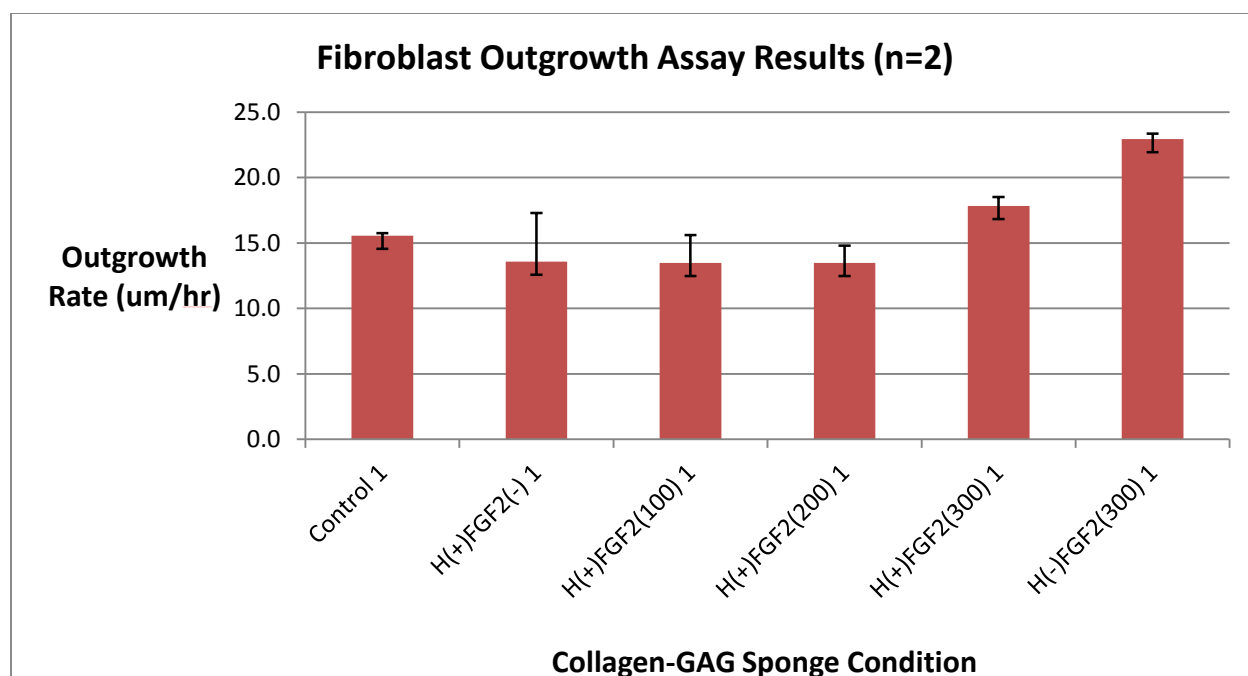
*Figure 41: This figure shows how measurements were made for each image and the format in which these measurements were saved. The yellow lines in the image represent the distance that was measured from the reference.*

Being consistent with image analysis techniques was essential for automating the data analysis process. Since the measurements being made for each image were saved to a CSV file with the same format, a program could be used to perform data analysis. The team utilized a program written in Python that read in the 60 CSV files provided from the image analysis and performed calculations to provide the outgrowth rates for each condition. The program also allowed the team to graph and determine whether there was linear correlation in each of the trials performed. Figure 42 represents the distance travelled from the reference for the control, heparin positive FGF-2 (300 ng/mL), and heparin negative FGF-2 (300 ng/mL).



*Figure 42: This figure shows the graphs of the distance the fibroblasts were located from the reference versus time. This allows for linear correlation assessment to determine if the graphs are linear as outgrowth rates have shown to be so.*

It was important to graph the data so that  $R^2$  values for each condition could be calculated. Since the desired value for each condition is an outgrowth rate, the  $R^2$  value should be somewhere between 0.9-1.0 indicating that the data is linear. Although not shown in Figure 42, linear correlation values were calculated for each condition. Fibroblast outgrowth rates of each condition were then calculated. This rate was calculated by subtracting the start position (distance from reference at T0) from the final position (distance from reference at T20). The fibroblast outgrowth rates for each condition are summarized in Figure 43.



*Figure 43: This figure summarizes the fibroblast outgrowth rates for each of the six conditions tested. The graph also includes an error bar showing the standard deviation of each data set. The outgrowth rates are an average of the two trials (n=2) performed for each condition.*

In order to better represent this data, Table 29 below summarizes the outgrowth rates, linear correlation values, and standard deviation for each of the six conditions.

<b>Table 29: This figure summarizes the fibroblast outgrowth rates for each of the six conditions tested. The table also includes standard deviation and linear correlation values for each condition.</b>			
	<b>Rate (um/hr)</b>	<b>Std Deviation</b>	<b>Linear Correlation</b>
<b>Control</b>	15.6	0.21	0.95
<b>H(+)-FGF2(-)</b>	13.6	3.73	0.95
<b>H(+)-FGF2(100)</b>	13.5	2.14	0.93
<b>H(+)-FGF2(200)</b>	13.5	1.32	0.89
<b>H(+)-FGF2(300)</b>	17.8	0.68	0.91
<b>H(-)-FGF2(300)</b>	22.9	0.41	0.93

It can be seen from Table 29 that heparin negative FGF-2 positive (300 ng/mL) had the largest outgrowth rate. This was followed by heparin positive FGF-2 positive (300 ng/mL),



indicating that FGF-2 at a concentration of 300 ng/mL had an increasing effect on fibroblast outgrowth rates when compared to the control.

### 7.3 Impact Analysis

This project analyzed the potential of a modified collagen scaffold to enhance vascularization, and it is therefore not confirmed that this design will be marketed or mass-produced in any way. The design incorporates the use of heparin and Fibroblast Growth Factor – 2 to promote the migration of endothelial cells toward the scaffold, and in turn indicate that the treatment would in fact stimulate the growth of blood vessels into the scaffold if tested *in vivo*. The following is the team’s analysis of the societal impact of the design

#### 7.3.1 Environmental Impact

This project has a negligible environmental impact. The project did not work with any materials that would affect the environment in production, use, or distribution. The primary components, Fibroblast Growth Factor-2, heparin sodium salt, and collagen type I, are not proven to have any hazardous effects that would raise concern for the environment.

#### 7.3.2 Economics

Economics in the medical industry deals with the efficiency, effectiveness, value, and behavior in the production and consumption of drugs, devices and treatment. The study of economics is crucial to determining that the overall benefits of a product will outweigh the costs, and that the producer is maximizing the benefits of the product within the available resources. To understand the entire scope of the economic impact of a product, the producer must consider the micro-economic evaluation of the product at the treatment level, the demand and regulation for the product, market equilibrium, and cost-benefit analysis – to name a few (Cheltenham, 1997). By FDA standards, this project worked with a cell – scaffold product, falling under the

“combination product” class of regulation: “a product composed of any combination of a drug and a device, a drug and a biological product or a drug, device, and biological product.” The modification that the team made to the scaffold is a step towards developing it into a market-ready product that would have the ability to treat patients with wounds incapable of healing naturally, and thus have a need for tissue engineered skin. While this project solely focused on the concept of promoting angiogenesis into the scaffold, if the complete product is eventually approved and reaches the point where it can be used effectively as treatment for patients, it has the ability to change the treatment and care for severe burn victims, among others. Overall, in a broader scope, the product would ultimately benefit patients, surgeons, and hospitals. It would save patients personal time and money, would save hospitals money, and would save surgeons invaluable time. Although growth factors and testing are expensive, long-term analysis would prove these benefits to validate any costs.

### 7.3.3 Societal Influence

The work that this project did alone could significantly impact the final scaffold product, increasing its likelihood of being approved and taken to market. It therefore has the ability to largely influence society, in the realm of tissue engineering research, and in the healthcare industry as a whole. If the scaffold has the ability to adequately promote vascularization into the treated wound bed of the patient, it could be used to facilitate the restoration of skin in a range of injuries, and therefore definitively impact the types of treatment that society would have access to. Within the clinical space of tissue engineering, the findings of this project could impact research across all areas of concentration – the need for adequate vascularization is not unique to skin alone; all organs and tissues in the body need proper vascularization in order to optimize

regeneration. If further testing could support that the methods in this project significantly promote angiogenesis, further application for similar research could be explored.

#### 7.3.4 Political Ramifications

The team does not expect any political ramifications to result from the work on this product.

#### 7.3.5 Ethical Concerns

Due to the inherent nature of cellular and tissue engineering, there are ethical concerns; however, in regards to this project, they are minimal. Experimentation utilized human Pulmonary artery endothelial cells (HPAECs) and bovine type I collagen. An ethical concern may be the sourcing of these materials, as they are human and animal, respectively. However, the HPAECs will not be a component of the final product, and therefore the ethical concern is negligible.

#### 7.3.6 Health and Safety Issue

Like stated previously, health and safety concerns are also inherent of any cellular and tissue engineering, medical research, and medical device production. This project worked on a product that is to ultimately be used as treatment for human patients. All materials are proven biocompatible, and able to be fully sterilized. The only component of this project that may pose an increased health and safety issue is the use of HPAECs, as they are a human cell source. The work with the HPAECs must be done with proper precaution and protection to ensure complete safety of the user.

#### 7.3.7 Manufacturability

The fabrication of the assay was kept to a relatively simple process. A protocol could be developed to enable outside researchers to easily develop the system independently. Additionally, a CAD drawing was made of the final design, and this could allow for mass-

production of the hardware component, making it marketable and distributable. The team cannot comment on the manufacturability of the  $\mu$ DERM scaffold as a whole; the project solely dealt with one component of it and did not observe nor practice fabrication of the entire product.

#### 7.3.8 Sustainability

Sustainability is based on the principle that the well-being of mankind is directly dependent on the environment, and as such the natural resources available to us must be maintained and protected to ensure lasting human health. None of the components of this project utilize natural resources that are in danger of over-consumption.

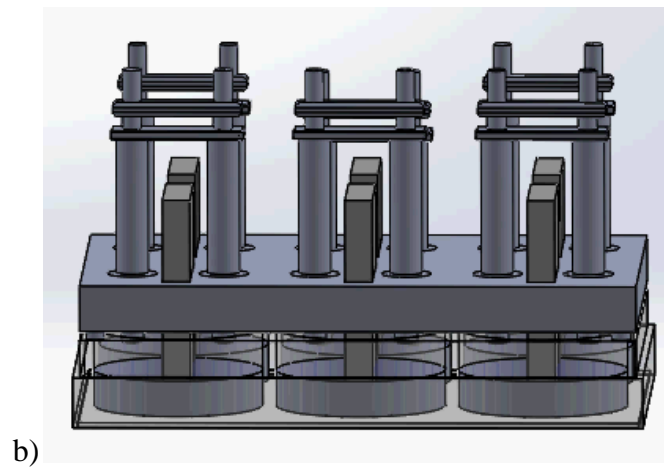
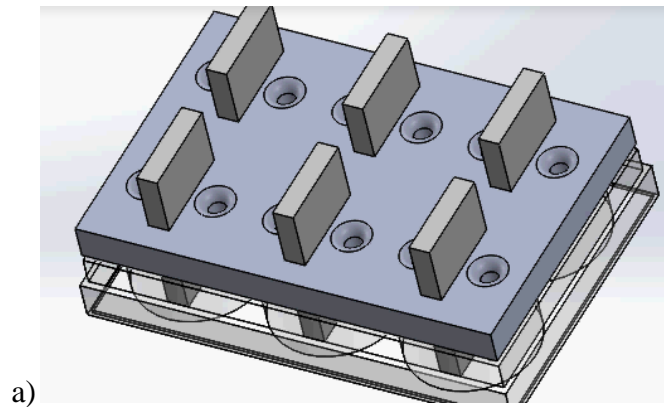
## 8.0 Conclusions and Recommendations

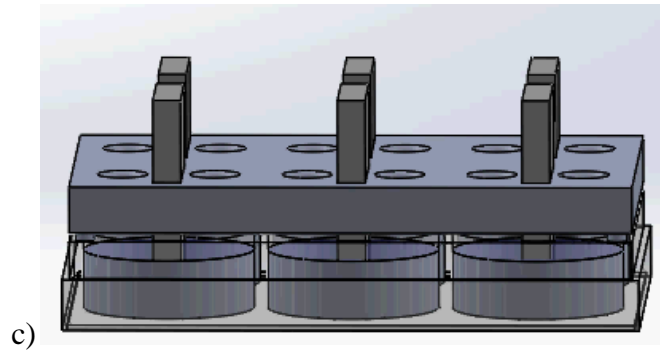
### 8.1 Conclusion

The project assessed vascularization of a collagen-GAG sponge modified with FGF-2 using an efficient assay developed to evaluate the effectiveness of the modification. The design incorporated the use of heparin and FGF-2 to promote the migration of fibroblast cells toward the scaffold, and in turn indicate that the treatment would potentially stimulate the growth of blood vessels into the scaffold if tested *in vivo*. The fibroblast outgrowth rates were highest when exposed to 300 ng/mL of FGF-2 compared to a non-heparinized, non-growth factor stimulated scaffold. These results can most likely be attributed to the initial burst of growth factor. Overall, the addition of heparin alone did not show significant effect on fibroblast outgrowth rates, and thus it is suggested that a future study collects data over a longer period of time to understand the potential long-term angiogenic effect that heparin could have on the scaffold. However, the results do suggest that the addition of FGF-2 to the  $\mu$ DERM would successfully enhance the scaffold's angiogenic ability, and thus overall increase its performance as an engineered skin substitute.

## 8.2 Future Recommendations

Future recommendations include design changes for the fabrication process in which the initial top mold that hold the glass slides has been modified for ease of use. As seen in Figure 44, the top part is brought closer to the six well plate and holes are spaced directly down into the wells to increase ease of use.





*Figure 44: The new design process with a customized top part for ease of use when creating PDMS wells. a) Shows the addition of the top part and glass slides to the 6-well plate b) shows the addition of media through the upper channels using a pipette c) shows the removal of pipettes*

The use of specific holes to add PDMS will also add to the precision and even coating of the PDMS in the well. Additionally, the decrease in materials needed to create the top part and slides will decrease costs.

Future recommendations for experimental changes include lowering the initial cell seeding density and waiting twenty-four hours before barrier removal. Waiting twenty-four hours will allow for cells to come to confluence without, but not enough time for cells to over-grow. This will decrease the amount of outlier cells that, when the barrier is removed, float ahead and adhere just in front of the actual starting point. These outliers skew data and decrease image quality. Making the above changes will create more accurate experiments and increase the quality of the data.

Additional steps include incorporation of endothelial cells into the assay, in which the endothelial cells will be measured travelling toward the collagen-GAG sponge and the sponge. The team would also like to look at the sponge via histological analysis at the end of the assay so as to determine the amount of migration and viability of endothelial in the scaffold. The team

predicts that they will see endothelial cell migration into the scaffold and initial micro-sprouting due to angiogenesis.

The team would then like to confirm vascularization through incorporation of the modified collagen-GAG sponge into  $\mu$ DERM and evaluation of overall performance of the entire system and *in vivo* animal testing. The team would like to place  $\mu$ DERM at a wounds site on the skin of the rat and measure the graft take and tissue in-growth over a period of 14 days. Each day after day four, a different rat would have  $\mu$ DERM removed and the team would perform histological analysis on the scaffold to determine if vascularization is occurring. The expected outcome is that initially there will be no vascularization, but that after four days, once the endothelial cells have migrated from the wound bed into  $\mu$ DERM, angiogenesis will begin to take place. It is believed by the team that angiogenesis will begin to occur around days 4-6 due to background research on when granulation and neovascularization stages intersect (Dipietro et al., 2010). The team also chose a 14 day experiment because after angiogenesis occurs, there is still a need for tissue-remodeling wound contraction and extracellular matrix reorganization which occurs during the second week of wound healing (Clark et. al, 1999). Similar research has been done on the topic of *in vivo* testing of dermal matrix in the rat model where Richter et al has done research on both cadaveric and porcine dermal matrices (Richter et al., 2007). For this experiment a 1 mm thick piece of AlloDerm, ENDURAGen, and meshed ENDURAGen were placed on rats and then harvested at 4 and 8 weeks. The grafts were analyzed using histological quantification and immunocytochemistry. It was found that AlloDerm showed better tissue ingrowth and micro vascularization density than the other two grafts (Richter et al., 2007). While Richter et al has an 8 week experiment, the team has cut the experimental time by 6 weeks because of the addition of the growth factor, FGF-2, to the collagen-GAG sponge, which is

predicted to allow for faster tissue in-growth into the dermal portion of  $\mu$ DERM and coincidentally, an increased rate of angiogenesis.



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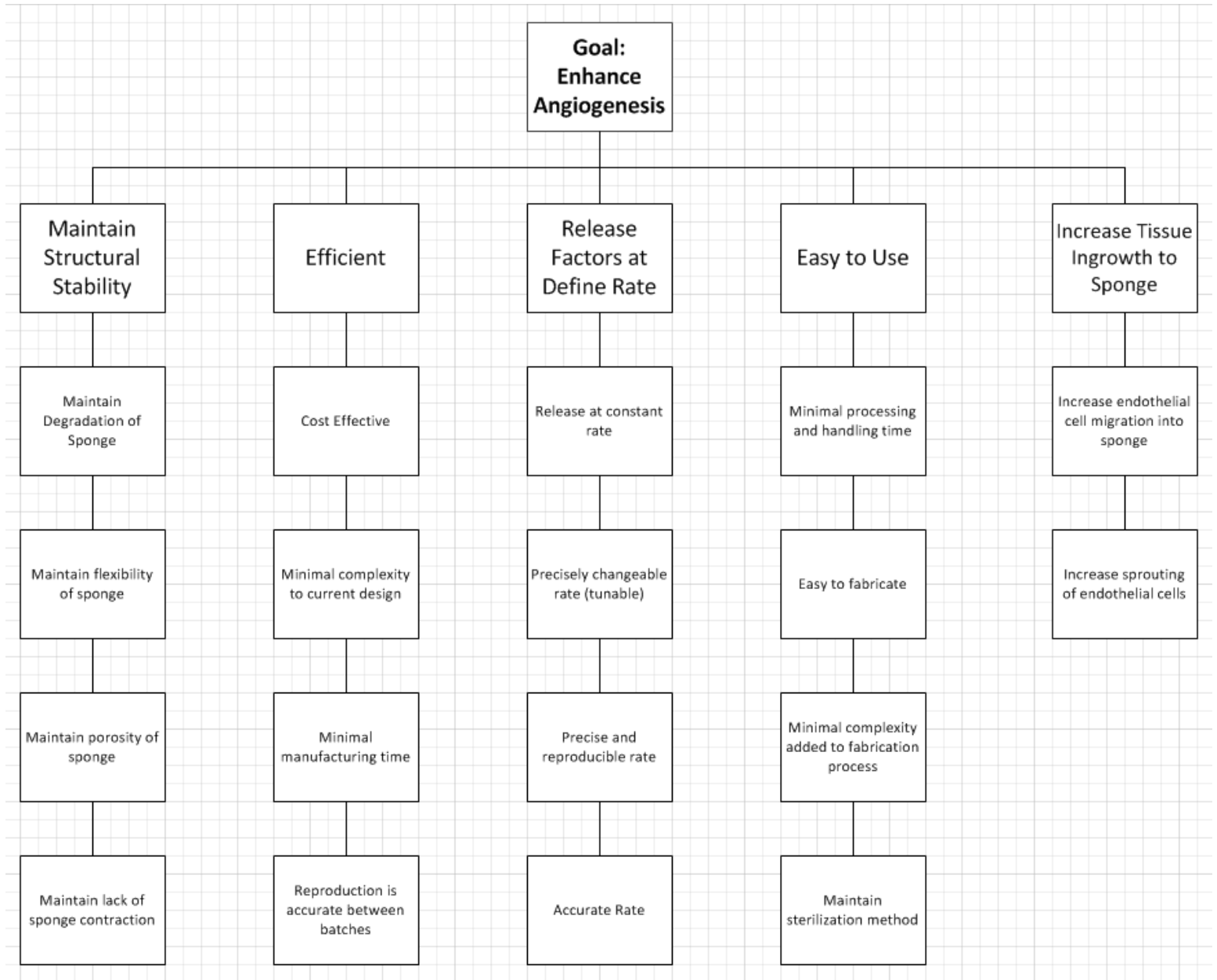
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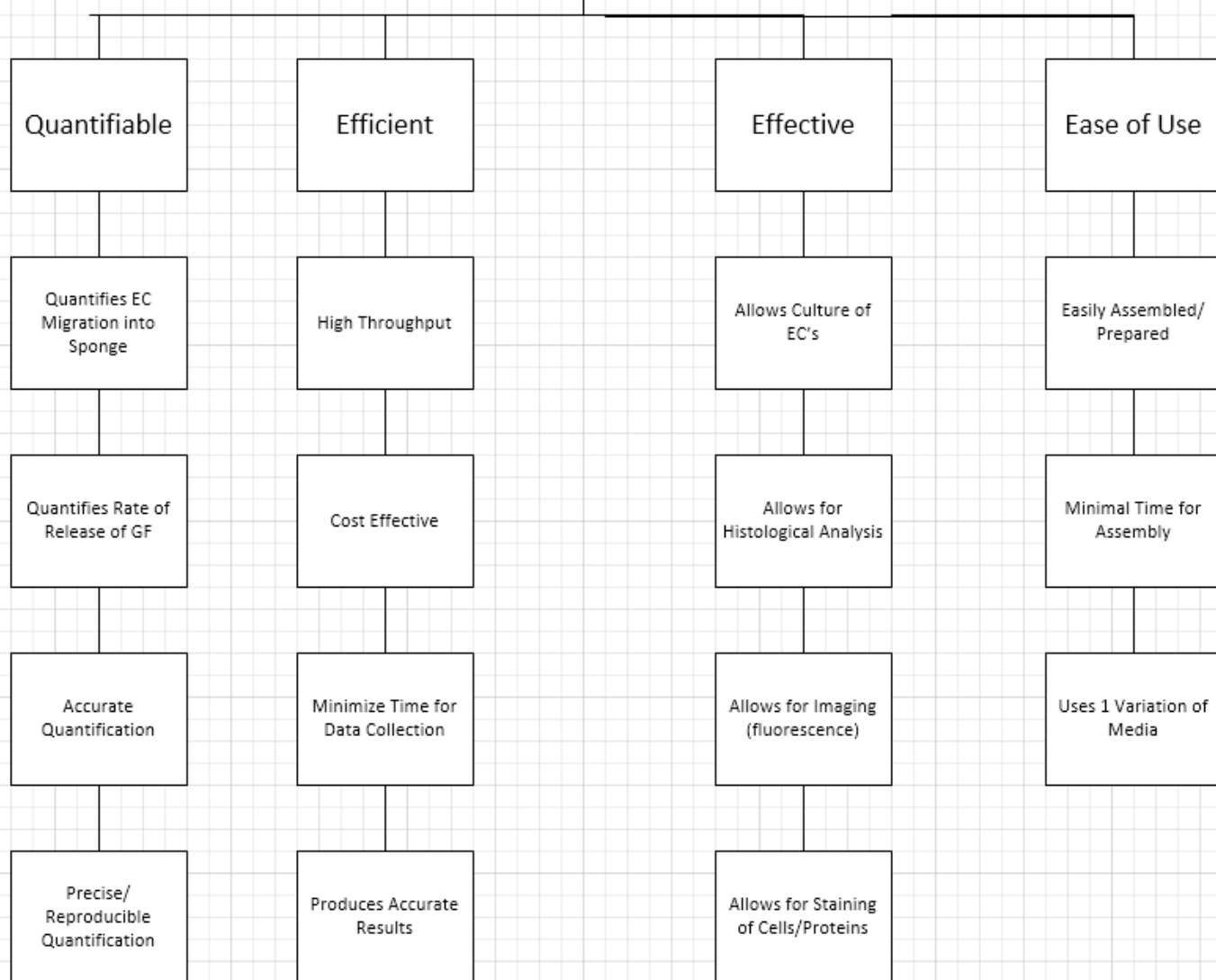
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## Appendix A: Objective Trees



**Assay To  
Asses EC  
Migration  
and Release  
Rate**



## Appendix B: Explanation of Objectives

### **Enhancing Angiogenesis:**

#### **I. Maintain scaffold stability**

The process by which the collagen sponge is fabricated by the team should yield the same stability as the current collagen sponge. Scaffold stability in this case refers to the measurement of degradation, flexibility and porosity. Degradation is defined as the rate at which the collagen sponge breaks down over a period of time. Flexibility can be sought as the scaffold should not be stiff. The current scaffold is very flexible (can be bent in half without tearing) and any addition to the design should maintain that property. The current scaffold also has a very specific porosity that through literature has been proven to be ideal; therefore, porosity should be maintained as close as possible to the porosity of the original sponge. Also, when the sponge is enhanced with growth factors it should not contract and maintain its stability as it currently stands

#### **II. Efficient**

The scaffold should also be efficient in the sense that it is cost effective, adds minimal complexity to the current design, has a minimal manufacturing time, and can be reproduced accurately by anyone in the lab. The scaffold must be cost effective in that it is affordable for the client. It also should be efficient in that the final design is not so complex that it takes away from the current sponge and should also be able to be manufactured as efficiently as it is currently. The scaffold should also be able to be fabricated accurately by performing its fabrication process.

### **III. Release factors at defined rate**

Factors that can be added to the scaffold to enhance angiogenesis should be released at a defined rate. Defined rate include release at a constant rate, a precisely tailored rate, a precise and reproducible rate and an accurate rate. The rate should be constant in that it releases a factor over a period of time in a consistent manner. The release rate should also be able to be tailored (changed) depending on the time period in which the factor needs to be released to effectively enhance angiogenesis. The release rate should also be precisely reproducible being that it is the same for each time the growth factor is incorporated. The release rate should also be accurate to the time period of growth factor release in natural wound healing.

### **IV. Easy to use**

The scaffold should be easy to use in that there is minimal processing and handling time, an easy fabrication process, minimal complexity added to fabrication process, and maintains the sterilization method of the current design. Minimal processing and handling time is important in that it minimizes the chances of contamination during the process as well as minimizes the chances of the user making a mistake during fabrication. The fabrication process should also be easy for the user to fabricate in that the user shouldn't need to execute the process at odd hours of the day or for extremely lengthy period of time. The user should also be able to use equipment available to her/him and not rely too much on manual labor. There should also be minimal complexity added to the current fabrication process. Also, it would be ideal to use the same sterilization technique used for the current scaffold.



## **V) Increase Tissue Ingrowth to Scaffold**

For proper vascularization to occur there must be interaction between the epidermal and dermal layers of the scaffold. A key aspect to obtaining this phenomenon is the migration of endothelial cells into the collagen sponge (dermal layer). The design must allow for endothelial cells to migrate into the sponge. If migration occurs, blood vessel formation (vascularization) can be achieved through the initiation of “sprouting” of endothelial cells which could potentially be assessed in vivo.

### **Assay to Assess Efficiency of Design:**

#### **I. Efficient**

The assay must be efficient in the sense that it is high throughput. High throughput means that it collects the maximum amount of different data in one analysis. It must also be cost effective because there is a chance that the assay will have to be produced multiple times. The assay must also minimize the time for data collection to make it easier for the user as well as reduce the time in which contamination can occur. The assay must also produce accurate results that are also precise and reproducible.

#### **II. Quantifiable**

The assay must also be quantifiable because if it is not there is no way to compare the designs efficacy to the actual stages of wound healing. The assay must be able to quantify the rate of endothelial cell migration into the sponge and quantify the rate of controlled release of the growth factor. This quantification should produce results that are accurate to the expected rates as well as be precise and reproducible between trials.

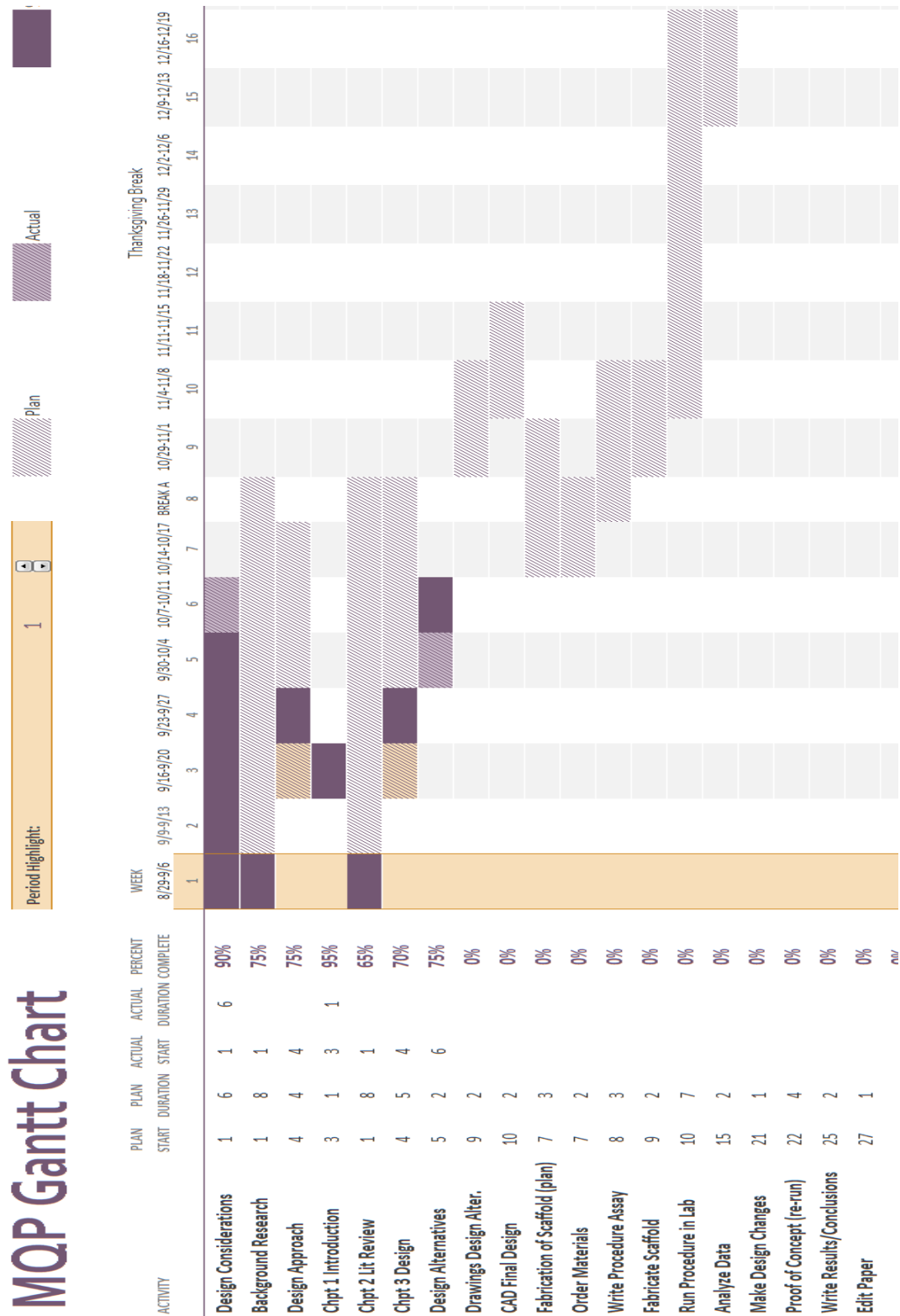
### **III. Effective**

The assay must be effective in that it performs all the necessary functions that would be ideal to examine the efficacy of our design. It must allow for culture of EC's as we are trying to quantify their migration rates. In order to view this migration, it is important that the assay allows for histological analysis/imaging of the sponge. The assay must also allow for staining of EC's so that their migration can be tracked in vitro.

### **IV. Ease of Use**

The assay must be easy to use for the user. It should be able to be easily assembled/prepared using a protocol written by the design team. The assay should allow for its assembly to be time efficient which will make it easier on the user as well as reduce chance of contamination. It should also be easy to use in that only one variation of medium is used and that it is readily available.

Appendix C: Team Management Gantt Chart



## Appendix D: Scoring Criteria

### Scoring Criteria Scaffold Design

#### **Increase Tissue Ingrowth:**

##### a. Increase EC ingrowth

- 4 points: 200% increase in ingrowth compared to control
- 3 points: 125% increase in ingrowth compared to control
- 2 points: 75% increase in ingrowth compared to control
- 1 point: 25% increase in ingrowth compared to control

##### b. Increase sprouting

- 4 points: 200% in capillary formation *in vivo* compared to control
- 3 points: 125% in capillary formation *in vivo* compared to control
- 2 points: 75% in capillary formation *in vivo* compared to control
- 1 point: 25% in capillary formation *in vivo* compared to control

#### **Release Factors at a Controlled Rate**

##### a. Precisely and reproducible rate

- 4 points: All trials within 5% of each other
- 3 points: All trials within 10% of each other
- 2 points: All trials within 15% of each other
- 1 point: All trials within > 20% of each other

##### b. Accurate rate

- 4 points: All trials within 5% of intended release
- 3 points: All trials within 10% of intended release
- 2 points: All trials within 15% of intended release

-1 point: All trials within > 20% of intended release

c. Constant rate

-4 points: Rate is constant at all time points

-3 points: Rate is constant at 75% of time points

-2 points: Rate is constant at 50% of time points

-1 point: Rate is constant at 25% of time points

d. Precisely tunable rate

-4 points: Tailored to unlimited number of rates

-3 points: Tailored to multiple rates

-2 points: Tailored to 1 rate

-1 point: Release rate can't be tailored

**Easy to Use:**

a. Minimal complexity added to fabrication process (current 9 steps)

-4 points: < 5 steps added

-3 points: 6-10 steps added

-2 points: 11-15 steps added

-1 point: > 16 steps added

b. Minimal processing and handling

-4 points: 1 person to handle

-3 points: 2 people to handle

-2 points: 3 people to handle

-1 point: 4+ people to handle

c. Easy to fabricate (components = materials and/or equipment)

- 4 points: < 5 components
- 3 points: 6-10 components
- 2 points: 11-15 components
- 1 point: > 16 components

**Efficient:**

a. Reproduction is accurate

- 4 points: All trials within 5% of control
- 3 points: All trials within 10% of control
- 2 points: All trials within 15% of control
- 1 point: All trials within > 20% of control

b. Minimal manufacturing time

- 4 points: < 3 hours
- 3 points: 3-6 hours
- 2 points: 6-9 hours
- 1 point: > 9 hours

c. Minimal complexity added to design (factor = component to achieve tissue ingrowth)

- 4 points: 1 “factor”
- 3 points: 2 “factors”
- 2 points: 3 “factors”
- 1 point: > 4 “factors”

d. Cost effective

- 4 points: Free-\$75
- 3 points: \$76-\$150

-2 points: \$151-\$225

-1 point: > \$226

## **Scoring Criteria Assay Design**

### **Effective:**

#### **a. Allow culture of EC's**

-4 points: 100% adhesion of ECs (cells are spread out and fully attached)

-3 points: 75% adhesion of ECs (cells are semi balled up)

-2 points: 50% adhesion of ECs (not all ECs present, ones that are look unhealthy)

-1 point: 25% adhesion of ECs (almost no ECs present)

#### **b. Allow for fluorescence imaging**

-4 points: <100% clear image

-3 points: 75% clear image

-2 points: 50% clear image

-1 point: >25% clear image

#### **c. Allow for staining of cells**

-4 points: 100% of cells are able to be stained

-3 points: 75% of cells are able to be stained

-2 points: 50% of cells are able to be stained

-1 point: 25% of cells are able to be stained

#### **d. Allow for histological analysis**

-4 points: allows for assay to be sliced without mechanical degradation

-3 points: allows for assay to be sliced with minimal degradation

-2 points: allows for assay to be sliced, but there is degradation

-1 point: assay is unable to be sliced due to degradation

## **Quantifiable**

### **a. Accurate quantification**

-4 points: 100% accurate from control

-3 points: 75% accurate from control

-2 points: 50% accurate from control

-1 point: 25% accurate from control

### **b. Quantifies EC ingrowth**

-4 points: able to quantify amount of EC's in scaffold

-3 points: able to get a rate of EC ingrowth

-2 points: able to get a rate of EC migration

-1 point: able to see movement, but unable to quantify it.

### **c. Precise/Reproducible quantification**

-4 points: 100% reproducible

-3 points: 75% reproducible

-2 points: 50% reproducible

-1 point: 25% reproducible

### **d. Quantify release rate**

-4 points: able to quantify release rate in scaffold

-3 points: able to quantify release rate outside of scaffold

-2 points: maybe able to get a number

-1 point: not able to quantify release rate



## **Efficient**

### a. Produce accurate results

-4 points: 100% accurate to control

-3 points: 75% accurate to control

-2 points: 50% accurate to control

-1 point: 25% accurate to control

### b. Produce reproducible results

-4 points: 100% reproducible

-3 points: 75% reproducible

-2 points: 50% reproducible

-1 point: 25% reproducible

### c. Minimize data collection time

-4 points: < 24 hours

-3 points: 24-48 hours

-2 points: 48-72 hours

-1 point: > 72 hours

### d. High throughput

-4 points: multi-use (3+ times), non-destructive

-3 points: non-destructive, two-use

-2 points: non-destructive, one-use

-1 point: destructive

### e. Cost effective

-4 points: < \$25

-3 points: \$25-50

-2 points: \$50-75

-1 point: > \$75

**Ease to Use:**

a. Easily assembled/prepared

-4 points: <5 parts

-3 points: 6-7 parts

-2 points: 7-9 parts

-1 point: 10+ parts

b. Minimal time for assembly

-4 points: < 3 hours

-3 points: 3-6 hours

-2 points: 6-9 hours

-1 point: > 9 hours

c. Use 1 variation of medium

-4 points: 1 variation

-3 points: 2 variations

-2 points: 3 variations

-1 point: > 4 variations

## Appendix E: Design Matrices

### Design Matrices for Scaffold Design

#### Increase Endothelial Cell Migration

MAX WEIGHTED SCORE = 100		RGD peptide	Weighted	Normalized	VEGF	Weighted	Normalized	micro-Topography	Weighted	Normalized	
Objectives:		Weight									
I. Increase Tissue Ingrowth											
a. Increase EC migration	4		3	12	0.12	4	16	0.16	1	4	0.04
b. Increase EC sprouting	4		1	4	0.04	4	16	0.16	1	4	0.04
II. Easy to Use											
a. Minimal complexity added	3	4	12	0.12	4	12	0.12	4	12	0.12	
b. Minimal processing	3	4	12	0.12	4	12	0.12	4	12	0.12	
c. Ease of fabrication	3	3	9	0.09	4	12	0.12	4	12	0.12	
III. Efficient											
a. Reproduction is accurate	2	4	8	0.08	3	6	0.06	2	4	0.04	
b. Minimal manufacturing time	2	1	2	0.02	2	4	0.04	4	8	0.08	
c. Minimal complexity to design	2	3	6	0.06	3	6	0.06	4	8	0.08	
d. Cost Effective	2	3	6	0.06	1	2	0.02	4	8	0.08	
TOTAL		26	71	0.71		86	0.86	28	72	0.72	
Constraints:											
Collagen-GAG Sponge		No				No			No		
Cell Compatible		No				No			No		
Biocompatible		No				No			No		
Sterilizable		No				No			No		
Maintain Mechanical Structure		No				No			No		
Budget (\$524)		No				Yes			No		
Time (28 weeks)		No				No			No		
Material Availability		No				No			No		
Lab Equipment Availability		No				No			No		
						</					

a. Reproduction is accurate	4	8	0.08	2		0	0	3	6	0.06
b. Minimal manufacturing time	1	2	0.02	2		0	0	3	6	0.06
c. Minimal complexity to design	3	6	0.06	2		0	0	3	6	0.06
d. Cost Effective	2	4	0.04	2		0	0	1	2	0.02
TOTAL	30	88	0.88			0	0	29	85	0.85
<b>Constraints:</b>										
Collagen-GAG Sponge	No				No			No		
Cell Compatible	No				No			No		
Biocompatible	No				No			No		
Sterilizable	No				No			No		
Maintain Mechanical Structure	No				No			No		
Budget (\$524)	No				Yes			No		
Time (28 weeks)	No				No			No		
Material Availability	No				No			No		
Lab Equipment Availability					No			No		

## Maintain Endothelial Cell Viability

<b>MAX WEIGHTED SCORE = 100</b>										
	<b>Weight</b>	<b>MSC's + EC's</b>	<b>Weighted</b>	<b>Normalized</b>	<b>Heparin</b>	<b>Weighted</b>	<b>Normalized</b>	<b>Mechanical Stretching</b>	<b>Weighted</b>	<b>Normalized</b>
<b>Objectives:</b>										
<b>I. Increase Tissue Ingrowth</b>										
a. Increase EC migration	4		0	0	2	8	0.08		0	0
b. Increase EC sprouting	4		0	0	2	8	0.08		0	0
<b>II. Easy to Use</b>										
a. Minimal complexity added	3		0	0	3	9	0.09		0	0
b. Minimal processing	3		0	0	3	9	0.09		0	0
c. Ease of fabrication	3		0	0	3	9	0.09		0	0
<b>III. Efficient</b>										
a. Reproduction is accurate	2		0	0	4	8	0.08		0	0
b. Minimal manufacturing time	2		0	0	3	6	0.06		0	0
c. Minimal complexity to design	2		0	0	3	6	0.06		0	0
d. Cost Effective	2		0	0	3	6	0.06		0	0
Total			0	0	26	69	0.69		0	0
<b>Constraints:</b>										
Collagen-GAG Sponge		No			No			No		
Cell Compatible		No			No			No		
Biocompatible		No			No			No		
Sterilizable		No			No			No		
Maintain Mechanical Structure		No			No			Yes		
Budget (\$524)		Yes			No			No		
Time (28 weeks)		Yes			No			Yes		
Material Availability		No			No			No		
Lab Equipment Availability		No			No			No		

		Hyaluronic Acid	Weighted	Normalized	5mM EDC crosslink	Weighted	Normalized	60mM EDC crosslink	Weighted	Normalized
<b>Objectives:</b>	<b>Weight</b>									
I. Increase Tissue Ingrowth										
a. Increase EC migration	4		0	0	2	8	0.08		0	0
b. Increase EC sprouting	4		0	0	1	4	0.04		0	0
II. Easy to Use										
a. Minimal complexity added	3		0	0	4	12	0.12		0	0
b. Minimal processing	3		0	0	4	12	0.12		0	0
c. Ease of fabrication	3		0	0	4	12	0.12		0	0
III. Efficient										
a. Reproduction is accurate	2		0	0	4	8	0.08		0	0
b. Minimal manufacturing time	2		0	0	4	8	0.08		0	0
c. Minimal complexity to design	2		0	0	2	4	0.04		0	0
d. Cost Effective	2		0	0	4	8	0.08		0	0
Total			0	0	29	76	0.76		0	0
<b>Constraints:</b>										
Collagen-GAG Sponge		Yes			No			No		
Cell Compatible		No			No			Yes		
Biocompatible		No			No			No		
Sterilizable		No			No			No		
Maintain Mechanical Structure		Yes			No			No		
Budget (\$524)		No			No			No		
Time (28 weeks)		No			No			No		
Material Availability		No			No			No		
Lab Equipment Availability		No			No			No		

## Mimics Angiogenesis

MAX WEIGHTED SCORE = 120		Hypoxia	Weighted	Normalized	Nitric Oxide	Weighted	Normalized	FG F-2	Weighted	Normalized	VE GF	Weighted	Normalized
<b>Objectives:</b>	<b>Weight</b>												
I. Increase Tissue Ingrowth													
a. Increase EC migration	4		0	0		0	0	4	16	0.1333333	4	16	0.1333333
b. Increase EC sprouting	4		0	0		0	0	4	16	0.1333333	4	16	0.1333333
II. Release Factors at Defined rate													
a. Precise and reproducible rate	3		0	0		0	0	3	9	0.075	3	9	0.075
b. accurate rate	3		0	0		0	0	3	9	0.075	3	9	0.075
c. Constant rate	3		0	0		0	0	3	9	0.075	2	6	0.05
d. Precisely tunable rate	3		0	0		0	0	2	6	0.05	2	6	0.05
III. Easy to Use			0	0		0	0						
a. Minimal complexity added	2		0	0		0	0	4	8	0.0666667	4	8	0.0666667

b. Minimal processing	2		0	0		0	0	4	8	0.0666 667	4	8	0.0666 667
c. Ease of fabrication	2		0	0		0	0	4	8	0.0666 667	4	8	0.0666 667
IV. Efficient													
a. Reproduction is accurate	1		0	0		0	0	4	4	0.0333 333	3	3	0.025 0.0083
b. Minimal manufacturing time	1		0	0		0	0	1	1	0.0083 333	1	1	0.0083 333
c. Minimal complexity to design	1		0	0		0	0	3	3	0.025 0.0166	3	3	0.025 0.0083
d. Cost Effective	1		0	0		0	0	2	2	0.0166 667	1	1	0.0083 333
Total								41	99	0.825		94	0.7833 3
Constraints:													
Collagen-GAG Sponge		No			No			No			No		
Cell Compatible		No			Yes			No			No		
Biocompatible		No			No			No			No		
Sterilizable		No			No			No			No		
Maintain Mechanical Structure		No			No			No			No		
Budget (\$524)		No			No			No			No		
Time (28 weeks)		No			No			No			No		
Material Availability		No			No			No			No		
Lab Equipment Availability		Yes			No			No			No		

		PDGF	Weighted	Normalized	TGF-beta	Weighted	Normalized	EGF	Weighted	Normalized
Objectives:		Weight								
I. Increase Tissue Ingrowth										
a. Increase EC migration	4	1	4	0.0333 333	1	4	0.0333 333	1	4	0.0333 333
b. Increase EC sprouting	4	3	12	0.1	2	8	0.0666 667	2	8	0.0666 667
II. Release Factors at Defined rate										
a. Precise and reproducible rate	3	3	9	0.075	3	9	0.075	3	9	0.075
b. accurate rate	3	3	9	0.075	3	9	0.075	3	9	0.075
c. Constant rate	3	2	6	0.05	3	9	0.075	3	9	0.075
d. Precisely tunable rate	3	2	6	0.05	2	6	0.05	2	6	0.05
III. Easy to Use										
a. Minimal complexity added	2	3	6	0.05 0.0666 667	2	4	0.0333 333 0.0666 667	3	6	0.05 0.0666 667
b. Minimal processing	2	4	8	0.0666 667	4	8	0.0666 667	4	8	0.0666 667
c. Ease of fabrication	2	4	8	0.0666 667	3	6	0.05	3	6	0.05
IV. Efficient										
a. Reproduction is accurate	1	3	3	0.025 0.0083 333	3	3	0.025 0.0166 667	3	3	0.025 0.0333 333
b. Minimal manufacturing time	1	1	1	0.025 0.0166 667	2	2	0.0166 667 0.0083 333	3	3	0.025 0.0083 333
c. Minimal complexity to design	1	3	3	0.025 0.0166 667	2	2	0.0166 667 0.0083 333	4	4	0.0333 333 0.0083 333
d. Cost Effective	1	2	2	0.0166 667 0.6416	1	1	0.0083 333 0.5916	1	1	0.0083 333 0.6333
Total			77	7		71	7		76	3
Constraints:										

Collagen-GAG Sponge	No	No	No
Cell Compatible	No	No	No
Biocompatible	No	No	No
Sterilizable	No	No	No
Maintain Mechanical Structure	No	No	No
Budget (\$524)	No	No	No
Time (28 weeks)	No	No	No
Material Availability	No	No	No
Lab Equipment Availability	No	No	No

## Controlled Release Rate

MAX WEIGHTED SCORE = 132		Gelatin Microspheres		Weighted	Normalized	PLGA Microspheres		Weighted	Normalized	Bind Factor to Sponge	Weighted	Normalized
Objectives:		Weight										
I. Release Factors at Defined rate												
a. Precise and reproducible rate	4	3	12	0.090909	1	4	16	0.121212	1	3	12	0.090909
b. accurate rate	4	4	16	0.121212	1	2	8	0.060606	1	2	8	0.060606
c. Constant rate	4	4	16	0.121212	1	1	4	0.030303	1	3	12	0.090909
d. Precisely tunable rate	4	4	16	0.121212	1	4	16	0.121212	1	1	4	0.030303
II. Easy to Use												
a. Minimal complexity added	3	2	6	0.045454	5	2	6	0.045454	5	4	12	0.090909
b. Minimal processing	3	4	12	0.090909	1	4	12	0.090909	1	4	12	0.090909
c. Ease of fabrication	3	3	9	0.068181	8	3	9	0.068181	8	4	12	0.090909
III. Efficient												
a. Reproduction is accurate	2	3	6	0.045454	5	4	8	0.060606	1	3	6	0.045454
b. Minimal manufacturing time	2	4	8	0.060606	1		0	0		3	6	0.045454
c. Minimal complexity to design	2	3	6	0.045454	5		0	0		4	8	0.060606
d. Cost Effective	2	4	8	0.060606	1	2	4	0.030303		2	4	0.030303
Total		38	115	0.87121		26	83	0.62879		33	96	0.72727
Constraints:												
Collagen-GAG Sponge		No				No				No		
Cell Compatible		No				No				No		
Biocompatible		No				No				No		
Sterilizable		No				No				No		
Maintain Mechanical Structure		No				No				No		
Budget (\$524)		No				No				No		
Time (28 weeks)		No				No				No		
Material Availability		No				No				No		
Lab Equipment Availability		No				No				No		

## Design Matrices for Assay Design:

### Allow for EC Culture

MAX WEIGHTED SCORE = 84										
	Weight	Collagen	Weighted	Normalized	Alginate	Weighted	Normalized	PDM	Weighted	Normalized
<b>Objectives:</b>										
I. Effective										
a. Allow culture of EC's	4	4	16	0.19047619	4	16	0.19047619	1	4	0.04761905
b. Allow for fluorescence imaging	4	3	12	0.14285714	4	16	0.19047619	3	12	0.14285714
c. Allow for staining of cells	4	4	16	0.19047619	4	16	0.19047619	4	16	0.19047619
d. Allow for histological analysis	4	3	12	0.14285714	3	12	0.14285714	4	16	0.19047619
III. Efficient										
a. Cost effective	3	4	8	0.0952381	3	6	0.07142857	4	8	0.0952381
IV. Ease of Use										
a. Easily assembled/prepared	2	4	4	0.04761905	4	4	0.04761905	4	4	0.04761905
b. Minimal time for assembly	2	1	1	0.01190476	3	3	0.03571429	1	1	0.01190476
c. Use 1 variation of media	2	4	4	0.04761905	4	4	0.04761905	4	4	0.04761905
Totals		27	73	<b>0.869048</b>	29	77	<b>0.916667</b>	25	65	<b>0.77381</b>
<b>Constraints:</b>										
Maintains scaffold structure		No			No			No		
Scaffold Imaging		No			No			No		
Budget (\$524)		No			No			No		
Time (28 weeks)		No			No			No		
Material Availability		No			No			No		
Lab Equipment Availability		No			No			No		
MAX WEIGHTED SCORE = 84										
	Weight	Chitosan	Weighted	Normalized	Agarose	Weighted	Normalized			
<b>Objectives:</b>										
I. Effective										
a. Allow culture of EC's	4	3	12	0.14285714	2	8	0.0952381			
b. Allow for fluorescence imaging	4	2	8	0.0952381	3	12	0.14285714			
c. Allow for staining of cells	4	4	16	0.19047619	4	16	0.19047619			
d. Allow for histological analysis	4	2	8	0.0952381	3	12	0.14285714			
III. Efficient										
a. Cost effective	3	3	6	0.07142857	4	8	0.0952381			
IV. Ease of Use										
a. Easily assembled/prepared	2	3	3	0.03571429	4	4	0.04761905			
b. Minimal time for assembly	2	2	2	0.02380952	4	4	0.04761905			
c. Use 1 variation of media	2	4	4	0.04761905	4	4	0.04761905			
Totals		23	59	<b>0.702381</b>	28	68	<b>0.809524</b>			



<b>Constraints:</b>		
Maintains scaffold structure	No	No
Scaffold Imaging	No	No
Budget (\$524)	No	No
Time (28 weeks)	No	No
Material Availability	No	No
Lab Equipment Availability	No	No

## Allow for non-destructive data collection

MAX WEIGHTED SCORE = 136													
Objectives:	Weight	topographical inward migration	Weighted	Normalized	topographical outward migration	Weighted	Normalized	gel inward migration	Weighted	Normalized	gel outward migration	Weighted	Normalized
I. Effective													
a. Allow culture of EC's	4	4	16	0.11764706	4	16	0.11764706	4	16	0.11764706	4	16	0.11764706
b. Allow for fluorescence imaging	4	4	16	0.11764706	4	16	0.11764706	3	12	0.08823529	3	12	0.08823529
c. Allow for staining of cells	4	4	16	0.11764706	4	16	0.11764706	3	12	0.08823529	3	12	0.08823529
d. Allow for histological analysis	4	2	8	0.05882353	2	8	0.05882353	3	12	0.08823529	3	12	0.08823529
II. Quantifiable													
a. Accurate quantification	3	3	9	0.06617647	3	9	0.06617647	2	6	0.04411765	2	6	0.04411765
b. Quantifies EC ingrowth	3	4	12	0.08823529	3	9	0.06617647	4	12	0.08823529	3	9	0.06617647
c. Precise/Reproducible Quantification	3	3	9	0.06617647	3	9	0.06617647	2	6	0.04411765	2	6	0.04411765
III. Efficient													
a. minimize data collection time	2	4	8	0.05882353	4	8	0.05882353	3	6	0.04411765	3	6	0.04411765
b. High throughput	2	4	8	0.05882353	4	8	0.05882353	3	6	0.04411765	3	6	0.04411765
c. Cost effective	2	3	6	0.04411765	3	6	0.04411765	3	6	0.04411765	3	6	0.04411765
IV. Ease of Use													
a. Easily assembled/prepared	1	4	4	0.02941176	4	4	0.02941176	3	3	0.02205882	3	3	0.02205882
b. Minimal time for assembly	1	4	4	0.02941176	4	4	0.02941176	4	4	0.02941176	4	4	0.02941176

c. Use 1 variation of media	1	4	4	0.029 4117 6	4	4	0.029 4117 6	4	4	0.029 4117 6	4	4	0.029 4117 6
				0.882 353			0.860 294			0.772 059			0.75
Totals		47	120		46	117		41	105		40	102	
Constraints:													
Maintains scaffold structure		No			No			No			No		
Scaffold Imaging		No			No			No			No		
Budget (\$524)		No			No			No			No		
Time (28 weeks)		No			No			No			No		
Material Availability		No			No			No			No		
Lab Equipment Availability		No			No			No			No		
Objectives:	Weight	topographical outward boundary migration	Weighted	Normalized	gel inward boundary migration	Weighted	Normalized	gel outward boundary migration	Weighted	Normalized			
I. Effective													
a. Allow culture of EC's	4	4	16	0.117 6470 6	4	16	0.117 6470 6	4	16	0.117 6470 6			
b. Allow for fluorescence imaging	4	4	16	0.117 6470 6	3	12	0.088 2352 9	3	12	0.088 2352 9			
c. Allow for staining of cells	4	4	16	0.117 6470 6	3	12	0.088 2352 9	3	12	0.088 2352 9			
d. Allow for histological analysis	4	2	8	0.058 8235 3	3	12	0.088 2352 9	3	12	0.088 2352 9			
II. Quantifiable													
a. Accurate quantification	3	4	12	0.088 2352 9	2	6	0.044 1176 5	2	6	0.044 1176 5			
b. Quantifies EC ingrowth	3	2	6	0.044 1176 5	2	6	0.044 1176 5	2	6	0.044 1176 5			
c. Precise/Reproducible Quantification	3	2	6	0.044 1176 5	2	6	0.044 1176 5	2	6	0.044 1176 5			
III. Efficient													
a. minimize data collection time	2	3	6	0.044 1176 5	2	4	0.029 4117 6	2	4	0.029 4117 6			
b. High throughput	2	4	8	0.058 8235 3	3	6	0.044 1176 5	3	6	0.044 1176 5			
c. Cost effective	2	3	6	0.044 1176 5	3	6	0.044 1176 5	3	6	0.044 1176 5			
IV. Ease of Use													
a. Easily assembled/prepared	1	4	4	0.029 4117 6	3	3	0.022 0588 2	3	3	0.022 0588 2			
b. Minimal time for assembly	1	4	4	0.029 4117 6	4	4	0.029 4117 6	4	4	0.029 4117 6			
c. Use 1 variation of media	1	4	4	0.029 4117 6	4	4	0.029 4117 6	4	4	0.029 4117 6			

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Totals	46	118	<b>0.867</b> <b>647</b>	44	116	<b>0.852</b> <b>941</b>	44	112	<b>0.823</b> <b>529</b>
<b>Constraints:</b>									
Maintains scaffold structure	No			No			No		
Scaffold Imaging	No			No			No		
Budget (\$524)	No			No			No		
Time (28 weeks)	No			No			No		
Material Availability	No			No			No		
Lab Equipment Availability	No			No			No		

## Allow for Quantification of Data

								Image J measure distance			
MAX WEIGHTED SCORE = 140			Manual Counting	Weighted	Normalized	MATLAB Program	Weighted	Normalized		Weighted	Normalized
Objectives:		Weight									
I. Quantifiable											
a. Accurate quantification	4	2	8	0.05714 286	4	16	0.11428 571	3	12	0.08571 429	
b. Quantifies EC ingrowth	4	3	12	0.08571 429	3	12	0.08571 429	4	16	0.11428 571	
c. Precise/Reproducible Quantification	4	2	8	0.05714 286	4	16	0.11428 571	3	12	0.08571 429	
d. Quantify release rate	4	1	4	0.02857 143	3	12	0.08571 429	2	8	0.05714 286	
II. Efficient											
a. Produce accurate results	3	2	6	0.04285 714	4	12	0.08571 429	3	9	0.06428 571	
b. Produce reproducible results	3	2	6	0.04285 714	4	12	0.08571 429	3	9	0.06428 571	
c. minimize data collection time	3	1	3	0.02142 857	4	12	0.08571 429	3	9	0.06428 571	
d. High throughput	3	1	3	0.02142 857	4	12	0.08571 429	3	9	0.06428 571	
e. Cost effective	3	4	12	0.08571 429	4	12	0.08571 429	4	12	0.08571 429	
III. Easy to Use											
a. Easily assembled/prepared	2	3	6	0.04285 714	2	4	0.02857 143	4	8	0.05714 286	
b. Minimal time for assembly	2	3	6	0.04285 714	2	4	0.02857 143	4	8	0.05714 286	
		24	74	0.52857 1	38	124	0.88571 4	36	112	0.8	
Constraints:											
Maintains scaffold structure		No			No			No			
Scaffold Imaging		No			No			No			
Budget (\$524)		No			No			No			
Time (28 weeks)		No			No			No			
Material Availability		No			No			No			
Lab Equipment Availability		No			No			No			

				ImageJ Quantify Fluorescence			
		Cell Profiler	Weighted	Normalized			Normalized
Objectives:	Weight						
I. Quantifiable							
a. Accurate quantification	4	3	12	0.08571429	3	12	0.08571429
b. Quantifies EC ingrowth	4	4	16	0.11428571	3	12	0.08571429
c. Precise/Reproducible Quantification	4	3	12	0.08571429	3	12	0.08571429
d. Quantify release rate	4	3	12	0.08571429	3	12	0.08571429
II. Efficient							
a. Produce accurate results	3	3	9	0.06428571	3	9	0.06428571
b. Produce reproducible results	3	3	9	0.06428571	3	9	0.06428571
c. minimize data collection time	3	3	9	0.06428571	3	9	0.06428571
d. High throughput	3	3	9	0.06428571	3	9	0.06428571
e. Cost effective	3	4	12	0.08571429	4	12	0.08571429
III. Easy to Use							
a. Easily assembled/prepared	2	3	6	0.04285714	4	8	0.05714286
b. Minimal time for assembly	2	3	6	0.04285714	4	8	0.05714286
		35	112	0.8	36	112	0.8
Constraints:							
Maintains scaffold structure		No				No	
Scaffold Imaging		No				No	
Budget (\$524)		No				No	
Time (28 weeks)		No				No	
Material Availability		No				No	
Lab Equipment Availability		No				No	

## Allows for Cellular Marking

MAX WEIGHTED SCORE = 152										
		GFP mark ed EC's	Weight ed	Normaliz ed	Hoec hst Staini ng	Weight ed	Normaliz ed	Alexa- Fluoro4 88 Stainin g	Weight ed	Normaliz ed
Objectives:	Weight									
I. Effective										
a. Allow culture of EC's	4	4	16	0.10526316	4	16	0.10526316	4	16	0.10526316
b. Allow for fluorescence imaging	4	4	16	0.10526316	4	16	0.10526316	4	16	0.10526316
c. Allow for staining of cells	4	4	16	0.10526316	4	16	0.10526316	4	16	0.10526316
d. Allow for histological analysis	4	4	16	0.10526316	4	16	0.10526316	4	16	0.10526316
II. Quantifiable										
a. Accurate quantification	3	4	12	0.07894737	3	9	0.05921053	4	12	0.07894737
b. Quantifies EC ingrowth	3	3	9	0.05921053	3	9	0.05921053	3	9	0.05921053

c. Precise/Reproducible Quantification	3	4	12	0.07894 737	3	9	0.05921 053	3	9	0.05921 053
III. Efficient										
a. Produce accurate results	2	4	8	0.05263 158	3	6	0.03947 368	4	8	0.05263 158
b. Produce reproducible results	2	4	8	0.05263 158	4	8	0.05263 158	4	8	0.05263 158
c. minimize data collection time	2	4	8	0.05263 158	3	6	0.03947 368	3	6	0.03947 368
d. High throughput	2	3	6	0.03947 368	3	6	0.03947 368	3	6	0.03947 368
e. Cost effective	2	1	2	0.01315 789	4	8	0.05263 158	2	4	0.02631 579
IV. Ease of Use										
a. Easily assembled/prepared	1	4	4	0.02631 579	3	3	0.01973 684	3	3	0.01973 684
b. Minimal time for assembly	1	4	4	0.02631 579	3	3	0.01973 684	3	3	0.01973 684
c. Use 1 variation of media	1	4	4	0.02631 579	4	4	0.02631 579	4	4	0.02631 579
Totals		55	141	<b>0.92763</b> <b>2</b>	52	135	<b>0.88815</b> <b>8</b>	52	136	<b>0.89473</b> <b>7</b>
<b>Constraints:</b>										
Maintains scaffold structure		No			No			No		
Scaffold Imaging		No			No			No		
Budget (\$524)		No			No			No		
Time (28 weeks)		No			No			No		
Material Availability		No			No			No		
Lab Equipment Availability		No			No			No		

## Appendix F: Experimental Procedures for Feasibility Testing

### Alginate Gel Procedure:

#### Materials

- 5g CaCl (Sigma-Aldrich)
- .5 liters and 100mL deionized H<sub>2</sub>O
- 2g Alginic Acid sodium salt from brown algae (Sigma-Aldrich)
- 2 500mL beakers
- Magnetic Plate and magnetic stirrer
- Plastic stirrer

#### Procedure

- Place CaCl in .5 liters of deionized water (use a 500mL beaker)
- Stir with plastic stirrer till forms a solution
- Place alginic acid sodium salt in 100mL deionized water (use a 500mL beaker)
- Place beaker with alginic acid on a magnetic plate. Place magnetic stirrer in beaker and allow to stir at medium speed for a ½ hour or until all the alginate clumps have dissolved, forming a viscous liquid.
- Pour viscous alginate liquid into the CaCl solution for long strings of gel or pipette droplets into the solution to get round drops of gel.
- Good for use immediately

### Collagen Gel:

#### Materials

- 0.8ml collagen at 10mg/mL in HCL (Pins Lab)
- 200microliters 5xDMEM (Pins Lab)
- 40microliters .1M NaOH
- 3 Syringes
- PDMS circle (~16mm radius)

#### Procedure

- Place ingredients and p35 plate on ice
  - Use separate syringes to measure out each ingredient
- Mix using the tip of the collagen syringe
  - Do not pipette mixture up and down
- Once well mixed, scrap mixture to corner of p35 plate and use pipette to remove mixture
- Pipette mixture on circle of PDMS
- Spread mixture to achieve the appropriate thickness
- Incubate overnight in 37 degree Celsius at 5%CO<sub>2</sub>, then use.

### **Agarose Gel Procedure:**

#### **Materials**

1g Agarose (SeaKem LE Agarose from LONZA)  
100mL deionized H<sub>2</sub>O  
Microwave  
500mL beaker  
Metal stirrer

#### **Procedure**

- a. Place agarose in 100ml deionized H<sub>2</sub>O (use 500ml beaker)
- b. Stir with metal stirrer till forms a solution
- c. Place in microwave for 1 minute, then take out and stir
- d. Repeat step C two more times
- e. Let beaker sit for 5min
- f. Pour gel into mold or coat molds by dipping into alginate
- g. Let sit for 15min, then use.

### **NIH/3T3 Fibroblast Medium Recipe:**

500ml DMEM 1X w/glucose + sodium pyruvate w/out glutamine (CellGro)

10% FBS (Ambady Lab)

5ml PenStrep 10000 U Pen./ml + 10000 ug Strep./ml (LONZA)

### **Protocol for Making Polydimethylsiloxane (PDMS):**

#### **Materials**

Sylgard Silicone Elastomer base (Ellsworth Adhesive #184 SYL ELAST)  
Sylgard Silicone Elastomer curing agent (Ellsworth Adhesive #184 SYL ELAST)  
Gloves (The elastomer reagents are sticky and may be difficult to wash off)

#### **Procedure**

- a. Weigh 10 parts Sylgard silicone elastomer base and 1 part Sylgard silicone elastomer curing agent. Note: DO NOT MIX THE STOCK SOLUTIONS!!! Use separate weighing materials for each reagent.
- b. Pour reagents together and thoroughly mix the elastomer base and curing agent.
- c. Pour the well mixed solution into your mold.
- d. De-gas the PDMS by putting it into a vacuum chamber for at least 1 hour (larger/thicker volumes of PDMS may require more time).
- e. After degassing, visually inspect the PDMS to ensure that there are no more bubbles. If there are, repeat steps 4 and 5.



- f. Cure the PDMS by placing the mold into an oven set for 60 °C for at least 1 hour (larger samples may require more time).

One important thing to keep in mind is that the uncured reagents are very tacky and can make a big mess of anything they contact (the degassing chamber, the scale used to weigh reagents).

Students should wear gloves when handling PDMS, be careful not to spill, and make sure they clean up the space and equipment they use when preparing PDMS. We keep “Goo Gone” in the lab for this reason...Students using PDMS will need access to the desiccating/vacuum chambers, a vacuum source, a scale (~ grams), weigh boats and an oven for curing.

### **Protocol for Fabrication of Gelatin Microspheres:**

1. Make an aqueous gelatin solution
  - a. Measure out 1g of gelatin on weigh paper using a scale and 9 ml of water in a centrifuge tube using a scale
  - b. Combine in centrifuge tube
2. Make a 40°C hot water bath
3. Heat gelatin solution in water bath at 40°C for 15 minutes inverting intermittently
4. Heat 150 mL olive oil in bottle to 40°C in container
5. Add gelatin solution to olive oil drop-wise using a 1000 µl micropipette in a circular motion to avoid placing droplets on top of one another.
6. Stir using a magnetic stirrer that completely covers the bottom of the container at 500 rpm for 10 minutes
7. Place in a refrigerator to decrease the temperature to 4°C (about 24 hours) which will allow the microspheres to form
8. Centrifuge top portion of olive oil (this should barely have any microspheres) at 5000 rpm for 5 minutes in centrifuge tubes
9. Pour off olive oil into a container to save for another use in making microspheres
10. Add 5-10mL of acetone to the oil and gelatin solution (Note: Work with acetone in fume hood)
11. Stir the solution at 60rpm for 30 seconds
12. Separate solution into centrifuge tubes
13. Centrifuge each tube at 5000rpm for 5 minutes
14. Discard supernatant from each tube
15. Add 40-50mL of acetone to each centrifuge tube
16. Invert each centrifuge tube vigorously, until the pellet is re-suspended
17. Centrifuge each tube at 5000rpm for 5 minutes
18. Remove supernatant from each tube



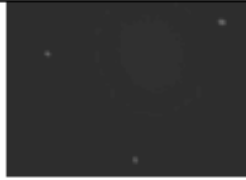
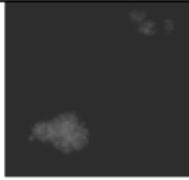

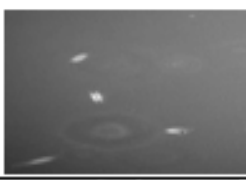





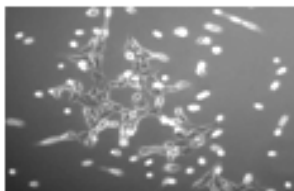
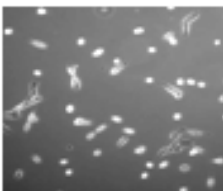
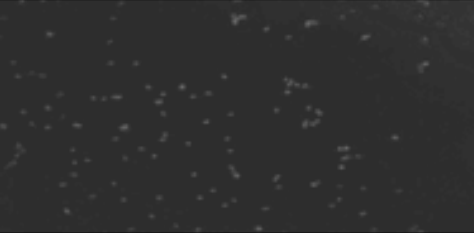


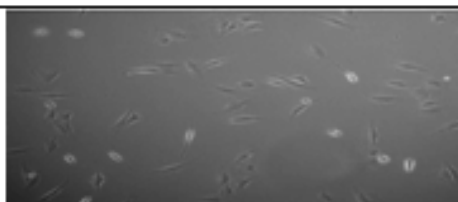
19. Repeat steps 14 to 17 until the oil is removed (microspheres will be able to move freely and should be the same color as the gelatin powder used to make microspheres)
20. Combine all microspheres into a beaker
21. Allow to dry in fume hood (about 2-3 hours)
22. Once dry move microspheres to centrifuge tube
23. Store in 4°C fridge

#### Washing and Sterilizing:

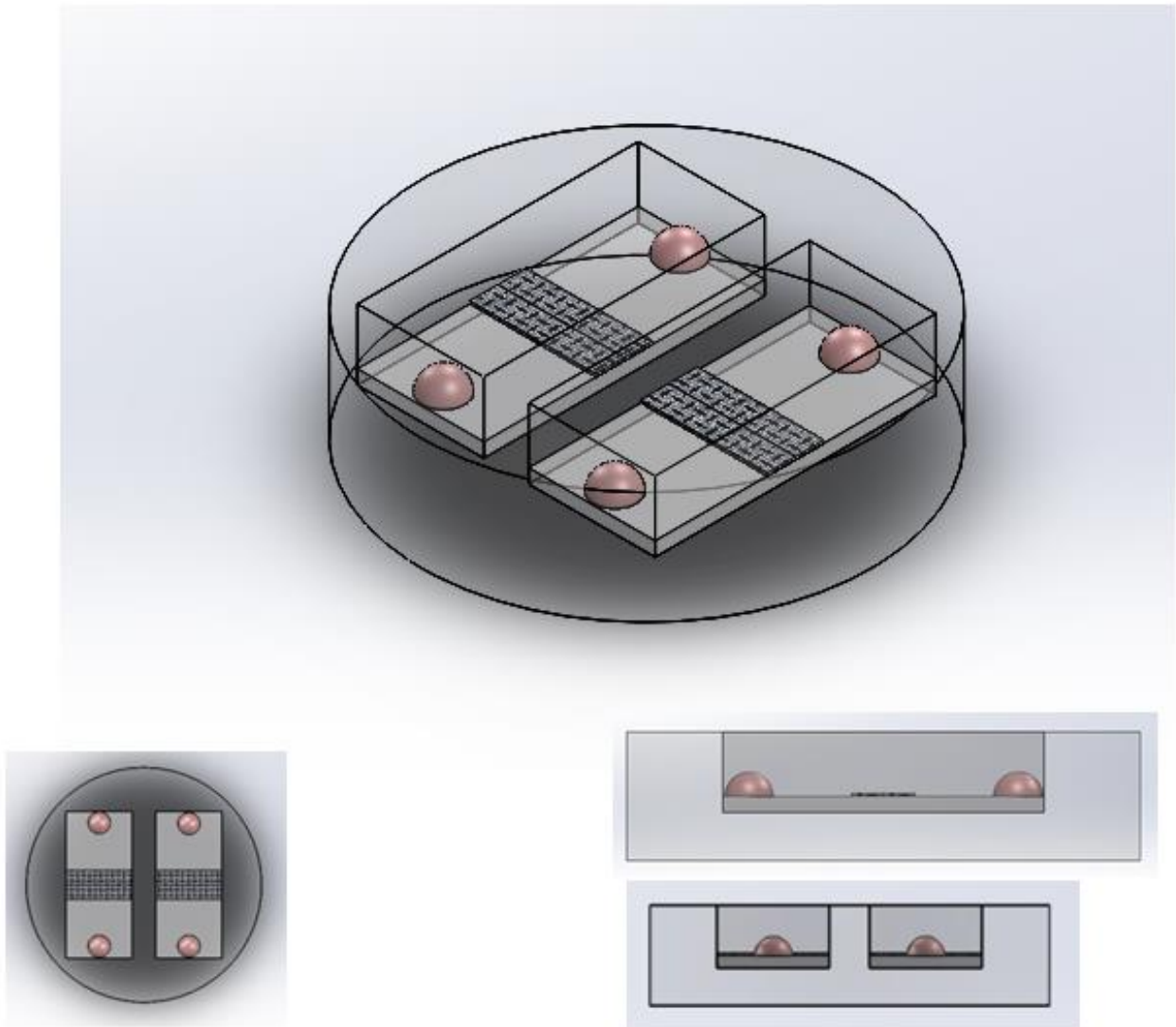
1. Measure out necessary amount of microspheres
2. Place in a 50mL centrifuge tube
3. Fill the tube to 45mL mark with room temperature IPA
4. Shake tube vigorously or vortex at highest speed
5. Place in centrifuge at 200 rcf for 5 minutes
6. Aspirate off IPA in culture hood
7. In same hood add cold DMEM to 45mL mark
8. Shake vigorously or vortex at highest speed
9. Place in centrifuge at 200 rcf for 5 minutes
10. Aspirate off DMEM in culture hood
11. In same hood add cold DMEM to 45 ml mark
12. Vortex microspheres at highest speed setting until microspheres appear dispersed.
13. Centrifuge at 200 rcf for 5 minutes
14. Aspirate off DMEM in culture hood
15. Add the necessary amount of warm full medium to microspheres for desired concentration
16. Shake tube vigorously.

*Note:* Solution can be stored after step 12. Complete steps 13 through 16 immediately before use with cells.

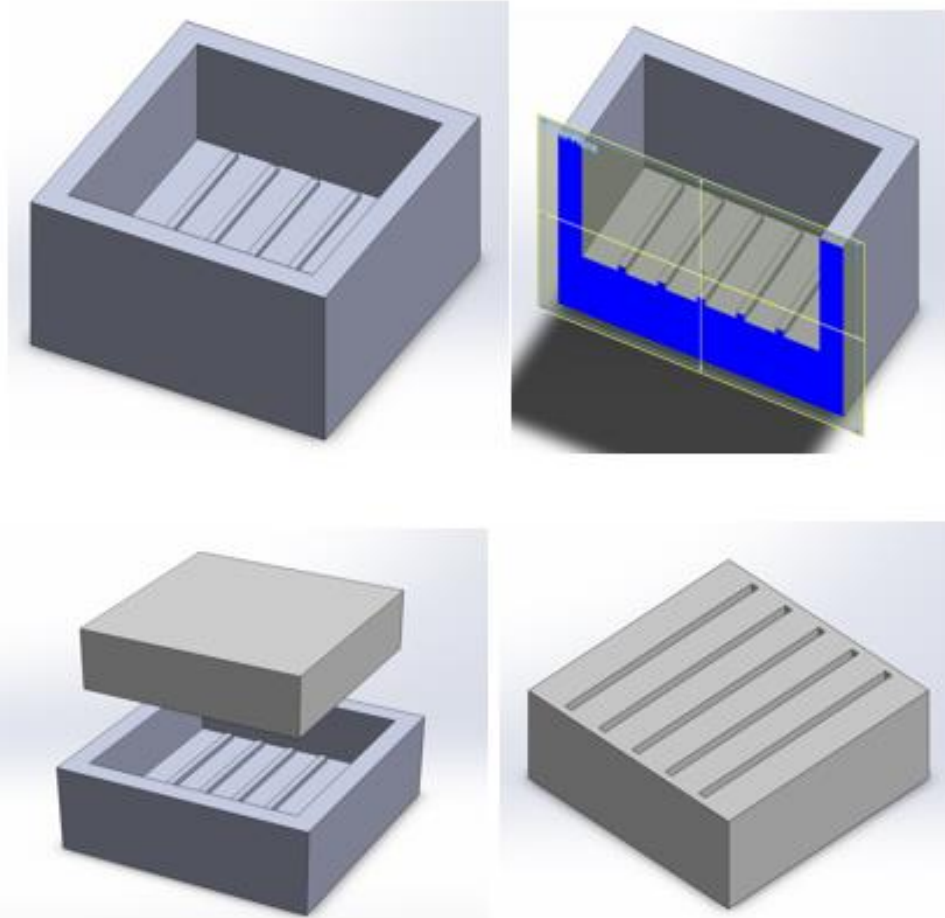
## Appendix G: Image Analysis of NIH/3T3 Fibroblasts on Gels for Feasibility Testing

.5mill NIH/3T3 cells/100uL plated on materials:	Inverted Microscope (10X)		Inverted Fluorescence Microscope (10X) (Hoechst staining)	
Alginate Poured at 1min after Heating				
Alginate Dipped at 1 min after Heating				
Collagen Coating (200ug/mL) on PDMS (thickness=5mm)				
Glass Slides				
PDMS (thickness= 5mm)				
Polystyrene				

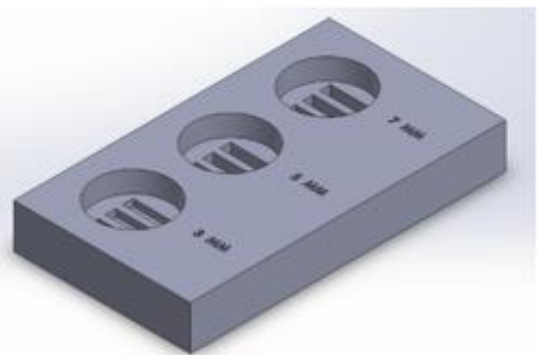
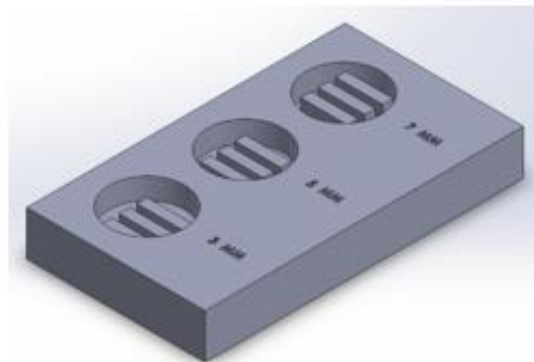
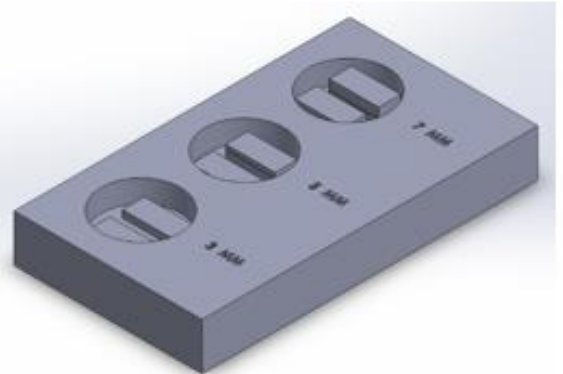
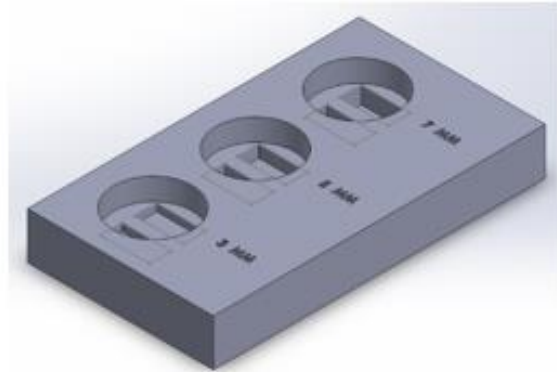
## Appendix H: Preliminary Assay Design Concept



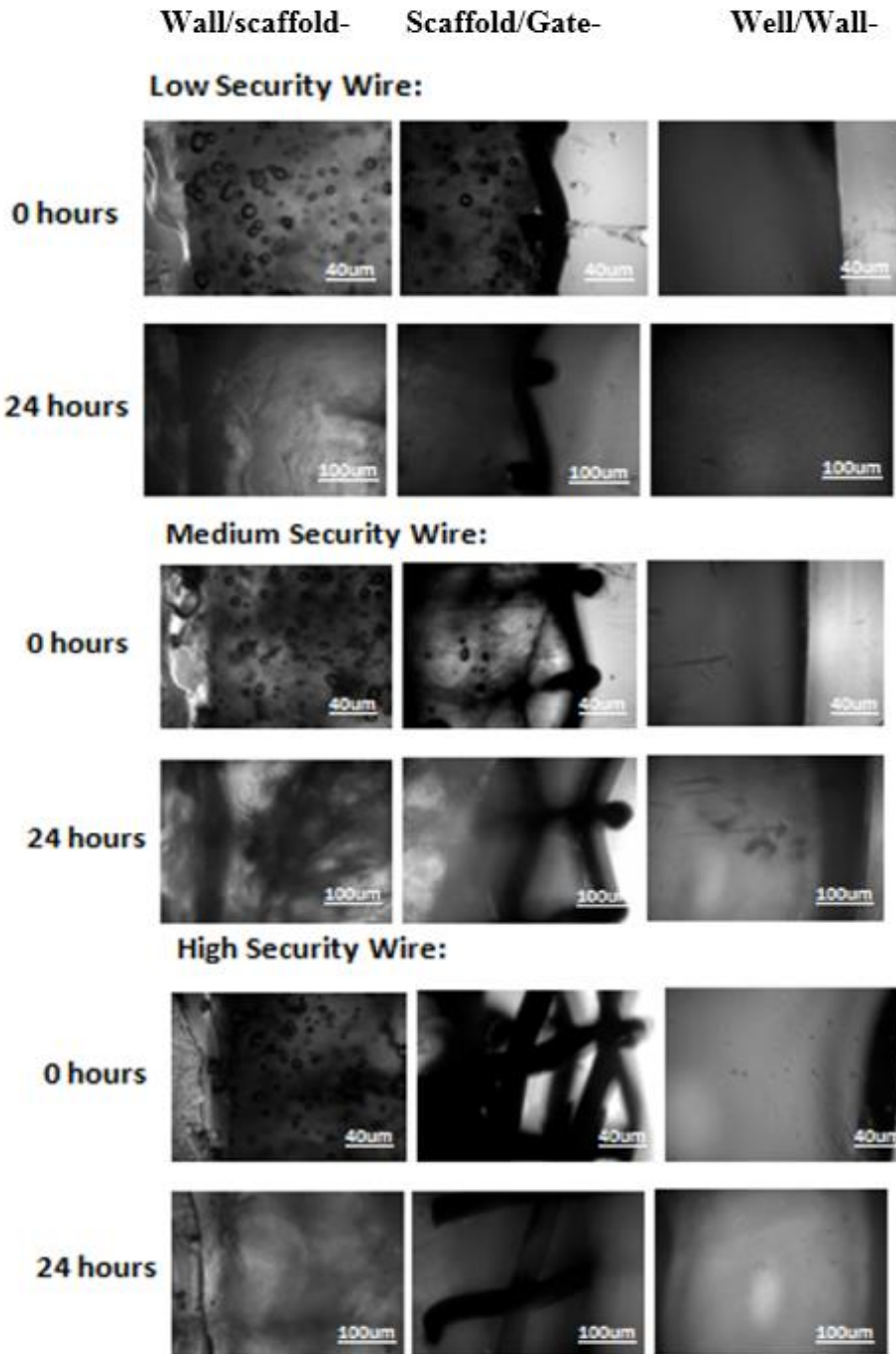
## Appendix I: Preliminary Assay Design: SolidWorks Model I



## Appendix J: Preliminary Assay Design: SolidWorks Model II



## Appendix K: Image Analysis for Containment of Scaffold



## Appendix L: Protocol for Heparin Immobilization on Collagen-GAG Sponge and FGF-2 Binding:

### Heparin Immobilization on Collagen-GAG Sponge

1. Collagen-GAG sponges are cut into circular scaffolds using a 9mm biopsy punch.
2. Sponges are incubated at room temperature in MES Buffer (0.5M & 8.0 pH) for at least 30 minutes.
3. A solution of MES Buffer containing Heparin Sodium Salt (Intestinal Mucosa) (CalBioTech), N-(3-Dimethylamideopropyl) ethylcarbodiimide hydrochloride (EDC) (Sigma-Aldrich), and N-Hydroxysuccinimide (NHS) (Sigma-Aldrich) was prepared.
  - Heparin Sodium Salt was added at 2% w/v MES Buffer Solution
  - EDC added at molar ratio of EDC:Heparin = 1:2
  - NHS added at molar ratio EDC: NHS = 0.6:1
4. Sponges are then incubated in the Heparin solution for 2 hours at room temperature to allow heparin binding to occur.
5. Heparinized sponges are transferred to a culture dish containing DPBS(-) and stored overnight in the incubator.

### FGF-2 Binding Procedure

1. FGF-2 solutions in the lab were available at a concentration of 100 µg/mL at a volume of 25 µL.
2. Solutions of FGF-2 are diluted by increasing the volume using calculated volumes of PBS (with 1% BSA w/v) to concentrations of 100 ng/mL, 200 ng/mL, and 300 ng/mL.
3. Sponges are incubated individually in a 100 µL solution of the desired concentration within a 24-well plate. The sponges are incubated for a total of 90 minutes at room temperature.
4. The sponges are then washed in DPBS(-) to rid unbound FGF-2 and placed overnight in individual wells of 100 µL of antibiotics to be sterilized.



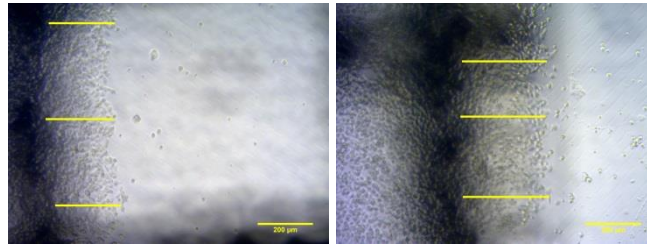
## Appendix M: Fibroblast Outgrowth Analysis for Six Conditions

Heparin Negative FGF-2 Negative (Control):

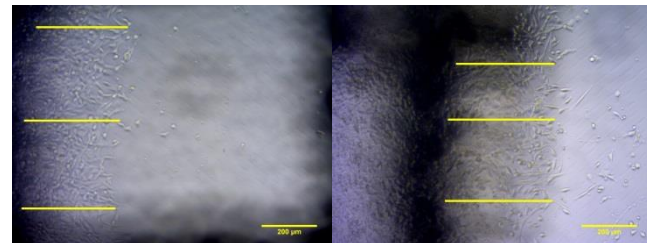
Trial 1:

Trial 2:

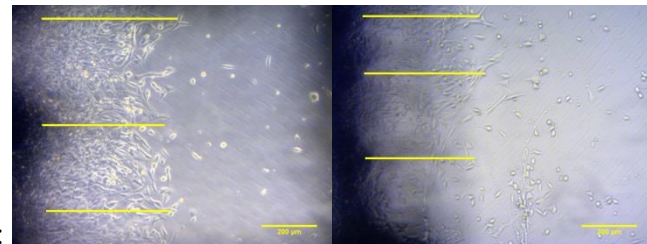
T0:



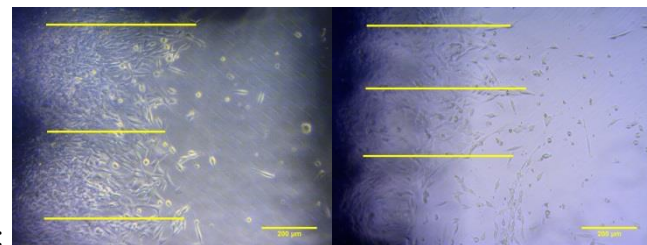
T8:

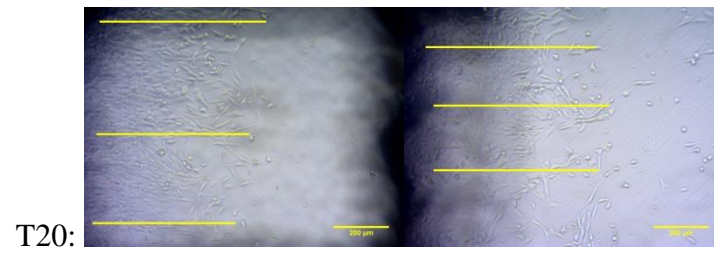


T12:



T16:

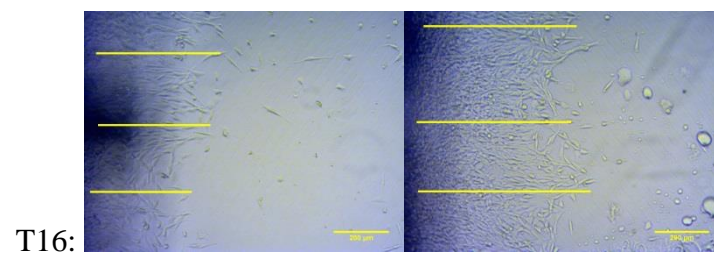
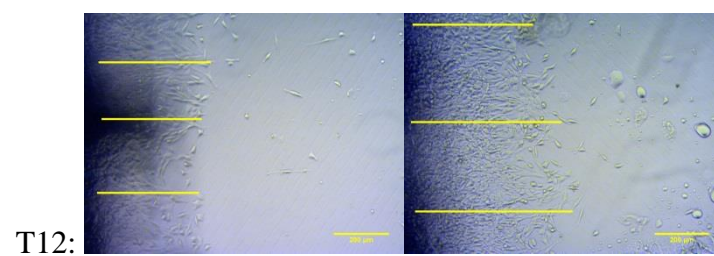
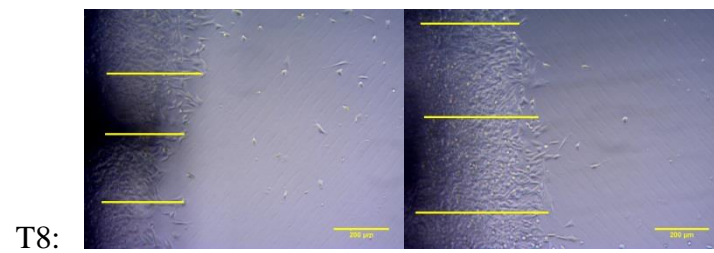
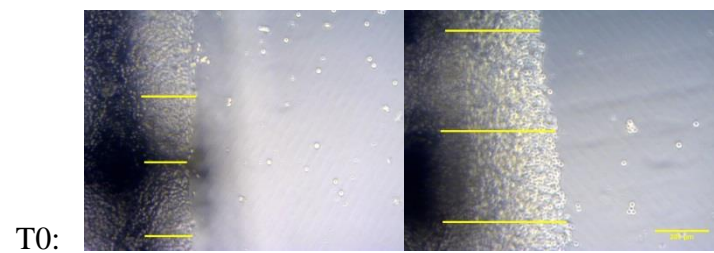


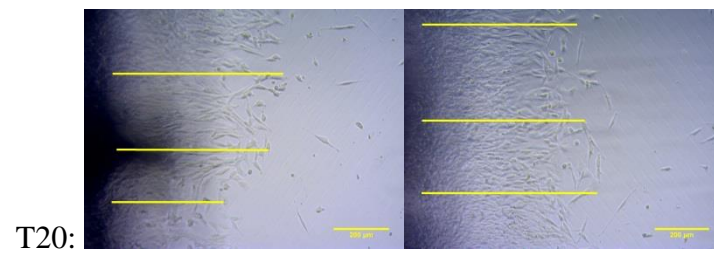


Heparin Positive FGF-2 Negative:

Trial 1:

Trial 2:

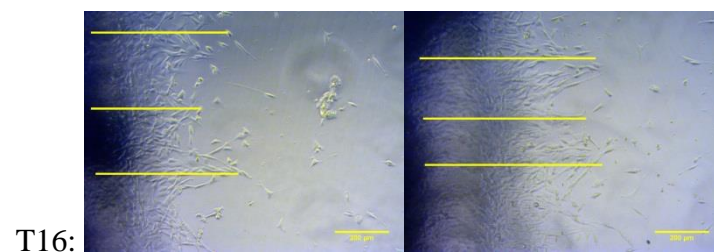
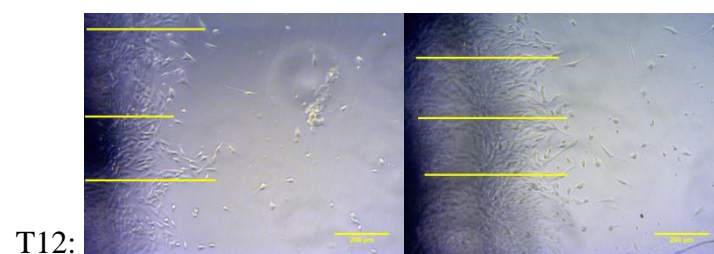
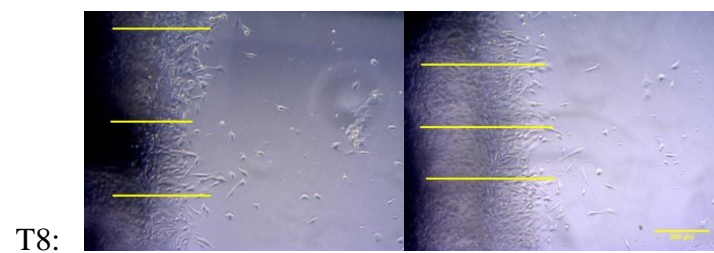
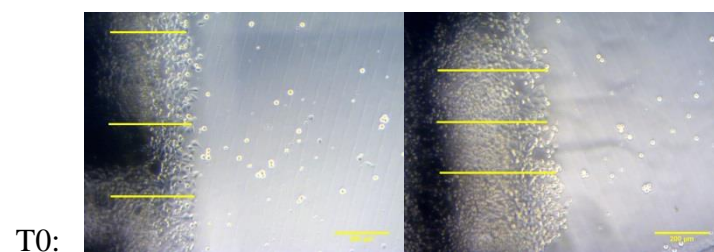


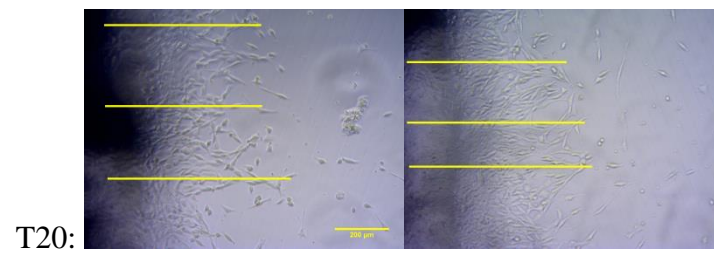


Heparin Positive FGF-2 (100 ng/mL):

Trial 1:

Trial 2:

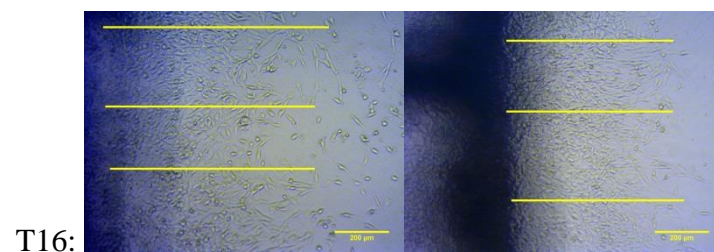
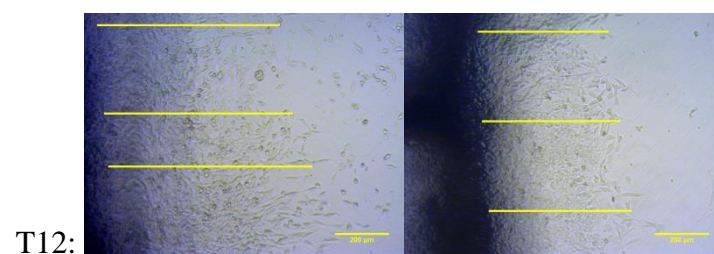
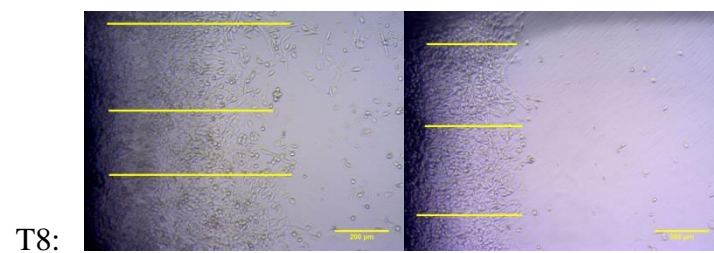
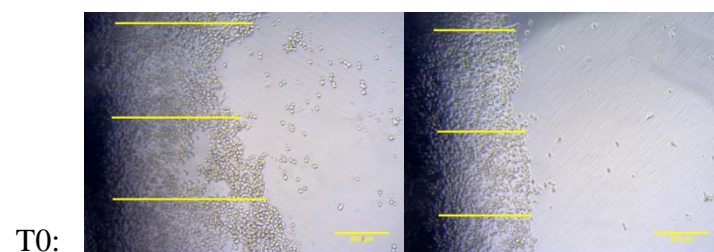




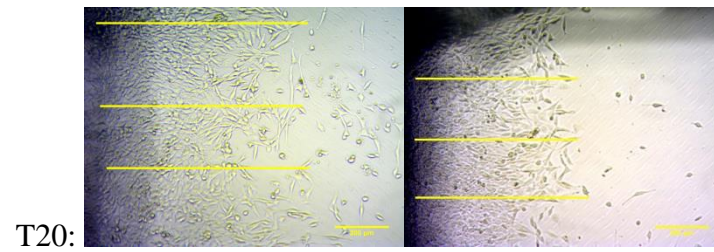
Heparin Positive FGF-2 (200 ng/mL):

Trial 1:

Trial 2:



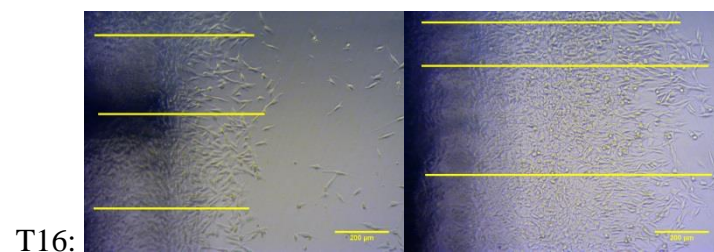
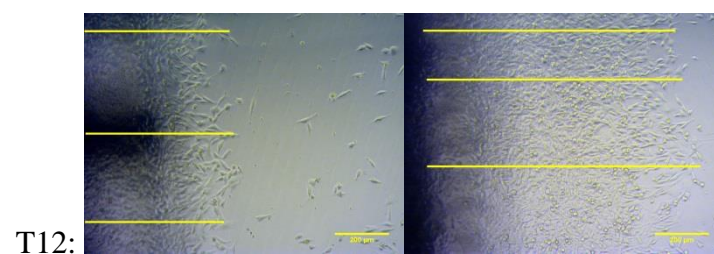
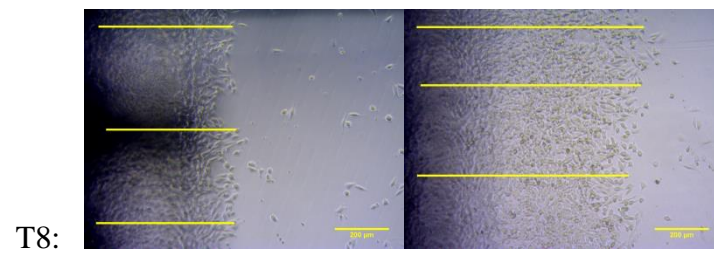
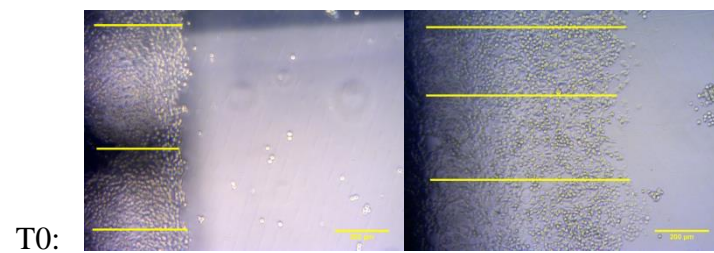


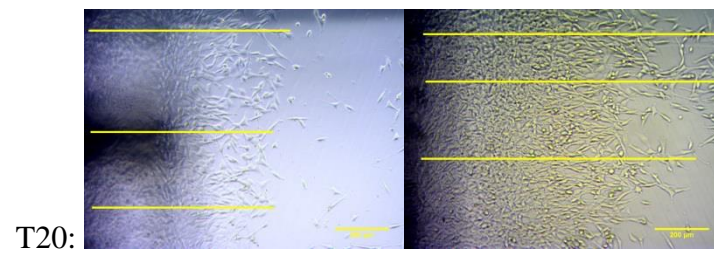


Heparin Positive FGF-2 (300 ng/mL):

Trial 1:

Trial 2:

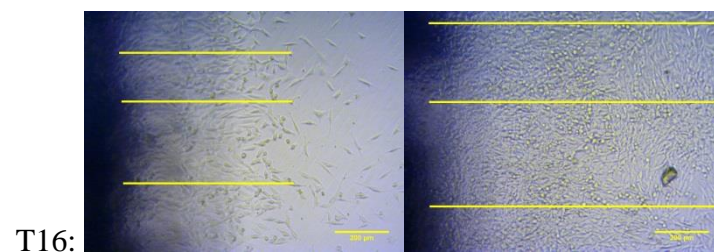
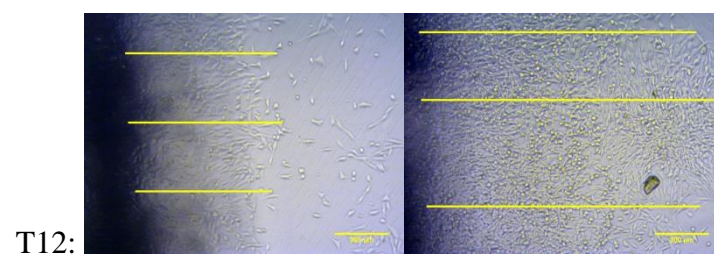
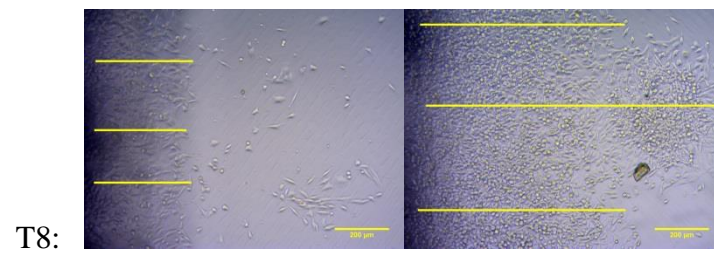
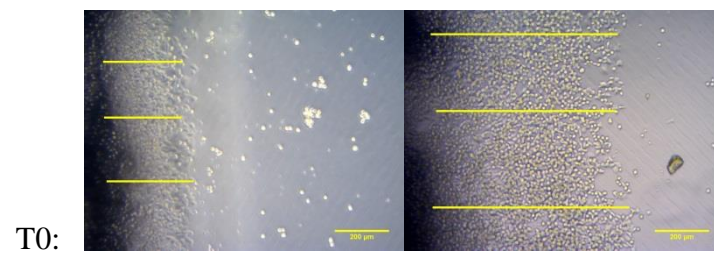




Heparin Negative FGF-2 (300 ng/mL):

Trial 1:

Trial 2:



T20:

